Water-Soluble Phosphate Prodrugs of 1-Propargyl-8-styrylxanthine Derivatives, A_{2A}-Selective Adenosine Receptor Antagonists†

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Water-soluble prodrugs of potent, A_{2A}-selective adenosine receptor (AR) antagonists were prepared. 8-(*m*-Bromostyryl)-3,7-dimethyl-1-propargylxanthine (BS-DMPX, **11**) and the analogous 8-(*m*-methoxystyryl)xanthine derivative (MS-DMPX, **5b**) were used as starting points. It was found that polar functional groups suitable for the attachment of a prodrug moiety were tolerated on the styryl ring and even better on the 3-substituent. 8-(*m*-Hydroxystyryl)-DMPX (**7**) and 3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-1-propargylxanthine (**5e**, MSX-2) were the most potent and A_{2A}-selective compounds and were selected for prodrug formation. For the preparation of **5e** a new ring-closure method was applied. Treatment of 6-amino-1-(3-hydroxypropyl)-5-(*m*-methoxycinnamoylamino)-3-propargyluracil with hexamethyldisilazane at high temperature resulted in higher yields of the target xanthine than the standard ring-closure procedure using sodium hydroxide. Phosphate prodrugs were prepared by classical phosphorylation using phosphorus oxychloride and alternatively by using a phosphoramidite method. Phosphates of the aliphatic alcohol **5e** could be obtained by both methods in similar yields. The phenolic compound **7**, however, could be phosphorylated only by using the phosphoramidite method. The disodium salts of the phosphate prodrugs exhibited high water solubility (8-(*m*-methoxystyryl)-7-methyl-3-[3-*O*-phosphatylpropyl]-1-propargylxanthine disodium salt, **9b**: 17 mM, 9 mg/mL). Prodrug **9b** was found to be stable in aqueous solution (pH 7) but readily cleaved by phosphatases to liberate **5e** (MSX-2). Compound **5e** showed high affinity for rat A_{2A} AR (K_i = 8 nM), human recombinant A_{2A} AR (K_i = 5 nM), and human native A_{2A} AR (K_i = 15 nM) and was highly selective versus rat A_1 AR (110-fold), human recombinant A_{2A} AR (500-fold), human A_{2B} AR (>2000-fold), and human A_3 AR (>2000-fold).

Introduction

Adenosine receptors (AR) play an important role in chemical signaling in both, peripheral and central, organs and tissues.[2] Four subtypes of ARs have been cloned: designated A_1, A_{2A}, A_{2B}, and A_3 AR. A_{2A} ARs are found in high density in certain brain regions, such as striatum, nucleus accumbens, and olfactory tubercle.[3] Adenosine exhibits high affinity to A_{2A} ARs, which appear to be tonically activated under physiological conditions.[4,5] A_{2A} AR antagonists have been proposed as novel therapeutics for Parkinson's disease[6] and may also be active as cognition enhancers, neuroprotectives, antiallergics, analgesics, and positive inotropics.[7,8]

A number of A_{2A}-selective AR antagonists have been developed comprising different classes of compounds, including xanthines and other bi- and tricyclic heterocyclic compounds.[8−10] Our group developed 3,7-dimethyl-1-propargyl-8-styrylxanthine (8-styryl-DMPX) derivatives as A_{2A}-selective AR antagonists.[11] A major problem of all high-affinity A_{2A} antagonists has been their low water solubility, which limits their usefulness especially for in vivo studies. The introduction of highly polar substituents, e.g. sulfonic or carboxylic acid groups, into potent, A_{2A}-selective antagonists resulted in a loss of receptor affinity.[12−14] Apparently, the A_{2A} AR does not tolerate such hydrophilic substituents. An alternative approach to increase water solubility would be the preparation of prodrugs, in which a polar moiety is attached to the drug but cleaved off in vivo to release the active compound. Ideally, such a prodrug should be stable in solution but rapidly degraded in vivo, preferably by an enzymatic reaction.[15] The A_{2A} antagonists used for prodrug formation need to have a suitable functional group for attachment of the prodrug moiety. As possible functional groups, we selected amino and hydroxy functions.[15] Thus, a number of hydroxyl- and amino-substituted derivatives of 8-styryl-DMPX were synthesized in order to investigate at which position the receptor would tolerate such a modification (see Scheme 1, Tables 2 and 4). Two hydroxyl derivatives were selected for prodrug formation, compounds **5e** and **7**. Phosphate prodrugs of those alcohols were prepared, for the concept of phosphate prodrugs is well-established: phosphoric acid esters are known to be stable in vitro but to be cleaved rapidly in vivo by phosphatases, which are ubiquitously found in the body.[16−19]

† Preliminary results were presented at the International Symposium on Adenosine and Adenine Nucleotides 1998 in Ferrara, Italy.[1]

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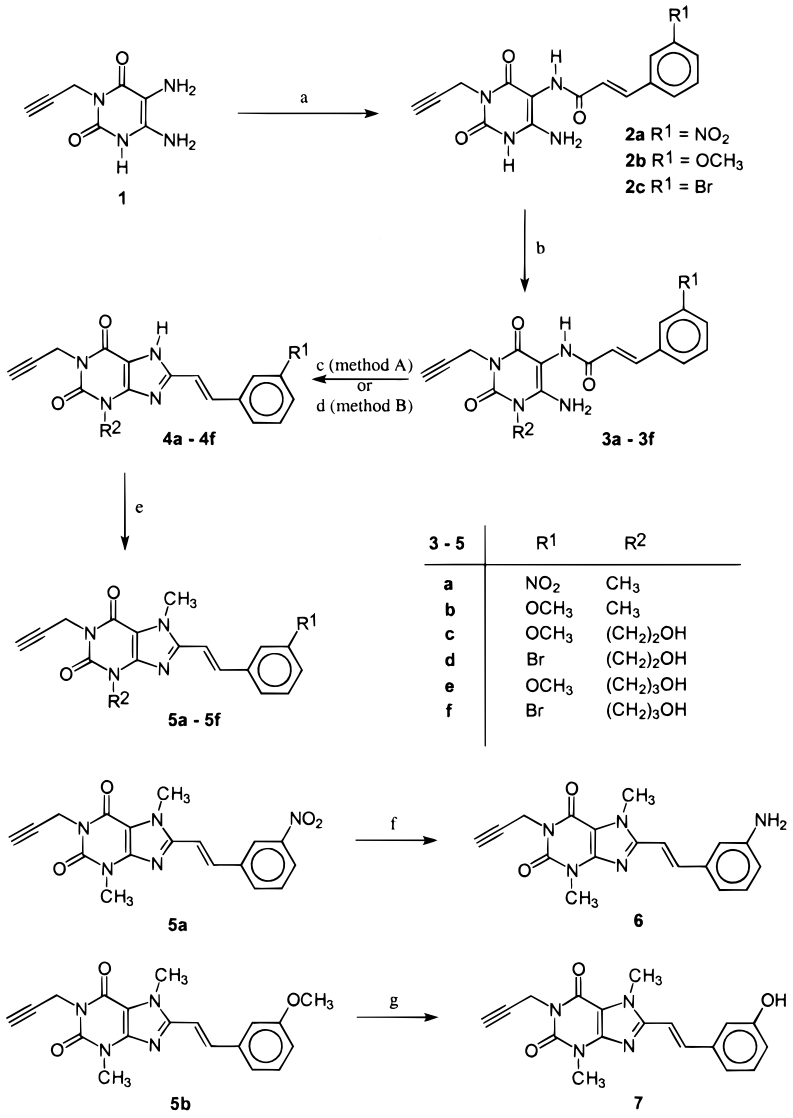
Scheme 1. Synthesis of A_{2A} Adenosine Receptor Antagonists Bearing Functional Groups for Prodrug Formation[a]



[a] (a) 3-Substituted cinnamic acid, EDC, MeOH, 4 h, rt; (b) R^2-X (X = Br, I), K_2CO_3, DMF, 24 h, 60 °C; (c) NaOH, MeOH, 3 h, 60 °C; (d) 1. HMDS, 24 h, 110 °C, 2. MeOH, H_2O; (e) CH_3I, K_2CO_3, DMF, 12 h, rt; (f) Zn, HOAc, 1.5 h, rt; (g) BBr_3, CH_2Cl_2, 3 h, 0 °C.

Results and Discussion

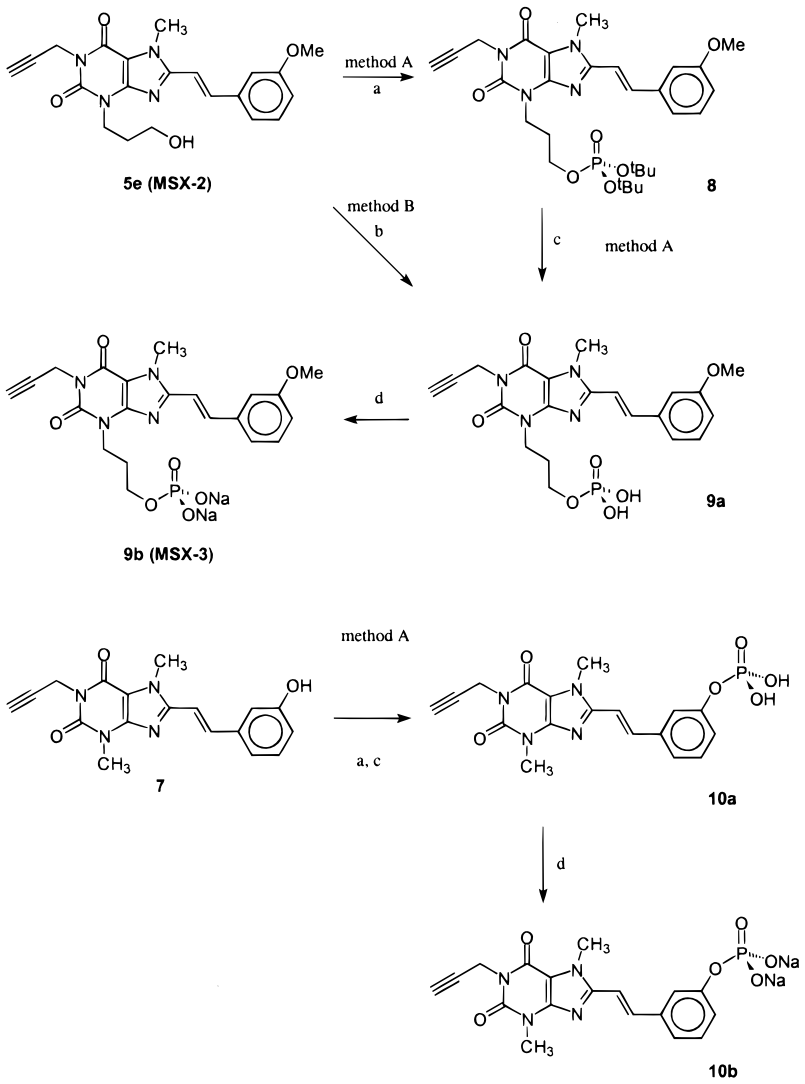
Chemistry. All products were prepared according to a recently developed synthesis for the preparation of xanthines with different substituents in the 1-, 3-, 7-, and 8-positions.[20] 5,6-Diamino-3-propargyluracil **(1)** was used as starting compound for all reactions (Scheme 1). For the preparation of carboxamido compounds **2a**−**2c**, diaminouracil derivative **1** was reacted with 3-bromo-, 3-methoxy-, or 3-nitrocinnamic acid, respectively, using a water-soluble carbodiimide as condensing agent.[11] Subsequent alkylation in the uracil 1-position was performed either with methyl iodide at room temperature or with 2-hydroxyethyl bromide or 3-hydroxypropyl bromide, respectively, at elevated temperatures. In all cases, potassium carbonate was used as a base in DMF yielding compounds **3a**−**3f**.

The ring-closure reaction to the xanthines **4a**−**4f** was achieved by treatment of **3a**−**3f** with dilute sodium hydroxide solution in methanol at reflux temperature (method A). For the synthesis of **4e**, we also used an alternative procedure for ring closure recently developed in our group.[21] Thus, compound **3e** was refluxed with hexamethyldisilazane for 24 h (method B). After hydrolysis, xanthine **4e** was obtained in 88% yield as compared to 55% obtained by the ring closure using sodium hydroxide (Table 1).

Subsequent methylation of compounds **4a**−**4f** in the 7-position with methyl iodide in the presence of potassium carbonate in dimethylformamide yielded the new 8-styrylxanthine derivatives **5a**−**5f**. The nitro group in nitrostyrylxanthine **5a** was reduced using zinc powder in acetic acid to obtain 8-(3-aminostyryl)-DMPX **(6)** (Scheme 1). 8-(3-Methoxystyryl)xanthine **(5b)** was converted to the corresponding 8-(3-hydroxystyryl)xanthine **(7)** by Lewis acid-catalyzed cleavage of the methoxy group (Scheme 1).

Phosphorylation of the hydroxy derivatives **5e** and **7** was initially attempted by treatment with phosphorus oxychloride. With the phenolic compound **7**, no reaction was observed at room temperature. Higher temperatures, however, led to a degradation of **7**. The more reactive primary aliphatic alcohol **5e** could be phosphorylated using phosphorus oxychloride in trimethyl phosphate under mild conditions (Scheme 2, method

Scheme 2. Synthesis of Phosphate Prodrugs of A_{2A}-Selective Adenosine Receptor Antagonists[a]



[a] (a) 1. $(^tBuO)_2P$-N$(Et)_2$, 1*H*-tetrazole, acetonitrile/CH_2Cl_2, 2 h, rt, 2. ^tBuOOH, 1 h, 0 °C; (b) $POCl_3$, $OP(OMe)_3$, 2 h, rt; (c) CF_3CO_2H, CH_2Cl_2, 1.5 h, rt; (d) Dowex WX4 (Na^+ form), H_2O, 24 h, rt.

B).[22] An alternative phosphorylation method was investigated for the preparation of aliphatic and aromatic phosphoric acid esters **9a** and **10a**. Thus, the phosphoramidite method, which is frequently used in oligonucleotide synthesis, was applied (Scheme 2). Di-*tert*-butyl *N*,*N*-diethylphosphoramidite was selected as a phosphitylating agent due to its favorable properties, i.e. a balanced stability−reactivity profile.[23] On activation with 1*H*-tetrazole, it reacted rapidly with the hydroxyl groups of **5e** and **7**. In situ oxidation of the resultant di-*tert*-butyl phosphite triester intermediates with *tert*-butyl hydroperoxide yielded di-*tert*-butyl phosphoric acid esters, such as **8** (Scheme 2, method A). Subsequent removal of the *tert*-butyl protecting groups was performed with trifluoroacetic acid in dichloromethane affording the phosphoric acid monoesters **9a** and **10a** in good yields. Cleavage of the *tert*-butyl phosphoric acid ester bond using hydrochloric acid, as reported in the literature,[23] was not successful in our hands. For the phosphorylation of compound **7**, the intermediate *tert*-butyl phosphoric acid triester was not isolated but directly deprotected with trifluoroacetic acid yielding phosphoric acid monoester **10a** (Scheme 2, method A). The total yields of the two different phosphorylation reaction pathways A and B for the preparation of **9a** were similar. The conversion of the phosphoric acid monoesters **9a** and **10a** into their sodium salts **9b** and **10b** was achieved by treatment of **9a** and **10a** with Dowex ion-exchange resin (Na^+ form) in aqueous solution (Scheme 2).

All analytical data were in accordance with the proposed structures. Typically, 7-methylated xanthine derivatives **5b**−**5f** exhibited lower melting points than the corresponding 7-unsubstituted xanthines (Table 1). The selected compound **5e** showed the lowest melting point of all investigated xanthine derivatives. In xanthines, such as caffeine, theophylline, and theobromine, lower melting points are associated with a lower tendency to form intermolecular aggregates and with increased water solubility despite higher lipophilicity.[24] NMR data are given in Tables 2 and 3 and as Supporting Information. Alkylation of uracil amides **2a**−**2c** at the uracil N1-nitrogen to yield compounds **3a**−**3f** caused the expected downfield shift of the neighboring 6-amino group from 6.1−6.2 to 6.7−6.8 ppm.[25] The disappearing of the signal for the 6-amino group in compounds **4a**−**4f** indicated the successful ring closure. Methylated compounds **5a**−**5f** showed the methyl signals at 4.04−

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

compd	yield (%)	formula	anal.[a]	M_r (g/mol)	mp (°C)
2a	80	$C_{16}H_{13}N_5O_5$	C, H, N	355.31	>250 dec
2b	70	$C_{17}H_{16}N_4O_4$	C, H, N	340.34	>250
3a	86	$C_{17}H_{15}N_5O_5$	C, H, N	369.34	>250
3b	81	$C_{18}H_{18}N_4O_4 \cdot 0.25H_2O$	C, H, N	358.87	241−243
3c	80	$C_{19}H_{20}N_4O_5$	C, H, N	384.39	221−222
3d	71	$C_{18}H_{17}BrN_4O_4$	C, H, N	433.26	178−179
3e	83	$C_{20}H_{22}N_4O_5 \cdot 0.5H_2O$	C, H, N	407.43	122−124
3f	73	$C_{19}H_{19}BrN_4O_4 \cdot 0.5H_2O$	C, H, N	456.30	134−137
4a	55	$C_{17}H_{13}N_5O_4$	C, H, N	351.32	>250
4b	52	$C_{18}H_{16}N_4O_3$	C, H, N	336.35	>250
4c	56	$C_{19}H_{18}N_4O_4$	C, H, N	366.38	>250
4d	57	$C_{18}H_{15}BrN_4O_3 \cdot 0.25H_2O$	C, H, N[b]	419.75	>250
4e	55,[d] 88[e]	$C_{20}H_{20}N_4O_4$	C, H, N	380.40	>250
4f	69	$C_{19}H_{17}BrN_4O_3$	C, H, N	429.27	>250
5a	88	$C_{18}H_{15}N_5O_4$	C, H, N	365.35	>250
5b	90	$C_{19}H_{18}N_4O_3 \cdot 0.5H_2O$	C, H, N	350.38	220−221
5c	89	$C_{20}H_{20}N_4O_4$	C, H, N	380.40	211−212
5d	81	$C_{19}H_{17}BrN_4O_3$	C, H, N	429.27	203−205
5e	93	$C_{21}H_{22}N_4O_4$	C, H, N	394.43	164−165
5f	83	$C_{20}H_{19}BrN_4O_3$	C, H, N	443.30	180−181
6	83	$C_{18}H_{17}N_5O_2$	C, H, N	335.36	251−252
7	78	$C_{18}H_{16}N_4O_3 \cdot 0.25H_2O$	C, H, N[c]	340.85	>250
8	71	$C_{29}H_{39}N_4O_7P$	C, H, N	586.62	158−160
9a	80,[f] 55[g]	$C_{21}H_{23}N_4O_7P$	C, H, N	474.40	193−195
9b	80	$C_{21}H_{21}N_4Na_2O_7P \cdot 0.5H_2O$	C, H, N	527.37	>200 dec
10a	65	$C_{18}H_{17}N_4O_6P \cdot 0.5H_2O$	C, H, N	425.34	>250
10b	90	$C_{18}H_{15}N_4Na_2O_6P \cdot 1.5H_2O$	C, H, N	487.34	>200 dec

[a] Elemental analyses were within ±0.4% of calculated values, unless otherwise noted. [b] Calcd, 13.35; found, 13.91. [c] Calcd, 16.43; found, 15.34. [d] Ring closure with NaOH (method A). [e] Ring closure with HMDS (method B). [f] Method A. [g] Method B.

4.12 ppm for the 7-methyl group, characteristic of 8-styryl(or aryl)-substituted xanthines.[20] Phosphorus-containing compounds were additionally characterized by their ^{31}P NMR spectra. Compound **8** showed one singlet at −9.5 ppm, which is typical of phosphoric acid triesters. After conversion to **9a**, the ^{31}P NMR signal is shifted to −1 ppm, characteristic of aliphatic phosphoric acid monoesters. Similarly, the ^{31}P NMR spectrum of **10a** showed a single signal at −6 ppm typical of aromatic phosphoric acid monoesters. The conversion of **9a** and **10a** to their disodium salts resulted in downfield shifts of the phosphorus signal of 2 ppm, from −6 to −4 ppm in compound **10b** and from −1 to +1 ppm in **9b (**Table 3).

Biological Evaluation. The synthesized 1-propargyl-8-styrylxanthine derivatives were investigated in radioligand binding studies at rat brain A_1 and A_{2A} AR using [^{3}H]N^6-cyclohexyladenosine (CHA) and [^{3}H]-2-[[[4-(carboxyethyl)phenyl]ethyl]amino]-5′-(*N*-ethylcarboxamido)adenosine (CGS21680), respectively, as radioligands. The selected compound **5e** (MSX-2) was additionally investigated for affinity to human recombinant A_1, A_{2A}, and A_3 ARs and to human post-mortem brain A_{2A} ARs, using [^{3}H]-2-chloro-N^6-cyclopentyladenosine (CCPA) for A_1, [^{3}H]CGS21680 for A_{2A}, and [^{3}H]-5′-(*N*-ethylcarboxamido)adenosine (NECA) for A_3 AR binding assays. Inhibition of agonist-induced stimulation of adenylate cyclase by **5e** was determined at human recombinant A_{2B} ARs.

Structure−Activity Relationships. 8-(*m*-Bromostyryl)-DMPX (BS-DMPX, **11**), a potent and selective A_{2A} AR antagonist,[11] was used as a reference compound. The analogous 8-(*m*-methoxystyryl)-DMPX (**5b**) exhibited similar affinity and selectivity (Table 4). Cleavage of **5b** to obtain 8-(*m*-hydroxystyryl)-DMPX (**7**) resulted in a ca. 2-fold reduction in A_{2A} AR affinity and selectivity versus A_1 AR (K_i = 21 nM, 45-fold selective). The analogous amino derivative **6** was less potent and less selective, compared to **7** (K_i = 42 nM). Since polar groups at the phenyl ring led to a reduction in A_{2A} affinity, a result which had also been observed earlier for other polar substituents,[12] it was decided to investigate derivatives bearing a hydroxyl group at the 3-substituent instead. 1-Propargyl-8-styrylxanthine derivatives were investigated, in which a 2-hydroxyethyl or 3-hydroxypropyl substitutent in the 3-position was combined with either *m*-bromo or *m*-methoxy substitution on the styryl ring.

Replacement of methyl by hydroxyethyl in the 3-position of BS-DMPX (**11**) or methoxystyryl-DMPX (**5b**), respectively, led to a 3-fold reduction in A_{2A} affinity. Since A_1 affinity remained virtually unaltered, A_{2A} selectivity was also reduced (compounds **5c** and **5d**). Hydroxypropyl derivatives, however, retained high A_{2A} affinity (compounds **5e** and **5f**). A_1 affinity of these compounds was slightly increased resulting in a somewhat lower A_{2A} selectivity of **5f** as compared to its 3-methyl analogue **11**.

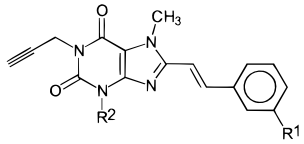
The most potent and selective compound of the present series, **5e**, designated MSX-2, and the hydroxystyryl derivative **7** were selected for prodrug formation. The synthesis of phosphate prodrug **9b** (designated MSX-3) from **5e** (MSX-2) was scaled up to obtain gram amounts for more detailed pharmacological investigations.

Characterization of 5e (MSX-2) and Prodrug 9b (MSX-3). The A_{2A} antagonist **5e** was investigated in radioligand binding assays at the human recombinant A_1, A_{2A}, and A_3 AR subtypes and in adenylate cyclase assays at human recombinant A_{2B} AR (Table 4). In addition, **5e** was investigated in binding assays at native human brain A_{2A} AR (Figure 1A). Compound **5e** was inactive at human A_{2B} and A_3 ARs in high concentrations (up to 10 μM). The compound was similarly potent at human A_{2A} AR (recombinant receptors, K_i = 5 nM; native receptors, K_i = 14.5 nM) as compared to rat A_{2A} AR (K_i = 8 nM) (Figure 1). Thus, compound **5e** is a highly selective A_{2A} AR antagonist in rat and humans with a selectivity versus A_1 AR greater than 100-fold.

The aqueous solution of the phosphate prodrug **9b** (MSX-3) of **5e** showed a pH value of 7. The water solubility of **9b** was determined by UV spectroscopy and found to be 9 mg/mL (17 mM), which is extraordinarily high compared to other high-affinity A_{2A} AR antagonists.[12] The prodrug **9b** proved to be stable in aqueous solution at room temperature for several hours. The addition of alkaline phosphatase, however, resulted in a rapid hydrolysis of the phosphate as detected by thin-layer chromatography.

Prodrug **9b** was also investigated in radioligand binding assays at rat A_1 and A_{2A} ARs. At A_{2A} AR, **9b** showed an apparent affinity of 150 nM (Figure 2). After preincubation with alkaline phosphatase the binding

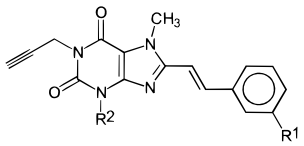
Table 2. Selected ^{1}H NMR Data of the Synthesized Compounds[a]



compd	R^1	R^2	^{1}H NMR δ (ppm)
5e	OCH_3	$(CH_2)_3OH$	1.89 (m, 2H, $CH_2CH_2CH_2$), 3.13 (t, J = 2.4 Hz, 1H, HC≡C), 3.52 (m, 2H, CH_2OH), 3.82 (s, 3H, OCH_3), 4.04 (s, 3H, N7−CH_3), 4.12 (m, 2H, N−CH_2), 4.52 (m, 3H, HC≡C−CH_2N, OH), 6.94 (m, 1H, $1H_{arom}$), 7.32−7.42 (m, 4H, $3H_{arom}$, CH=CHAr), 7.65 (d, 1H, J = 15.9 Hz, CH=CHAr)
7	OH	CH_3	3.09 (t, J = 2.2 Hz, 1H, HC≡C), 3.50 (s, 3H, N3−CH_3), 3.81 (s, 3H, N7−CH_3), 4.59 (d, J=2.2 Hz, 2H, HC≡C−CH_2N), 6.77 (m, 1H, H_{arom}), 7.12−7.28 (m, 4H, $3H_{arom}$, CH=CHAr), 7.58 (d, J = 15.5 Hz, 1H, CH=CHAr), 9.54 (s, 1H, OH)
8[b]	OCH_3	$(CH_2)_3OPO(O^tBu)_2$	1.51 (s, 18H, 2 O^tBu), 2.14−2.24 (m, 3H, HC≡C−, −CH_2−), 3.86 (s, 3H, N7−CH_3), 4.05 (s, 3H, OCH_3), 4.10−4.17 (m, 2H, N3−CH_2−), 4.25−4.32 (m, 2H, O−CH_2−), 4.79 (d, J = 2.6 Hz, 2H, −CH_2−N1), 6.88 (d, J = 15.7 Hz, 1H, −CH=CHAr), 6.89−7.37 (m, 4H, $4H_{arom}$), 7.79 (d, J = 15.7 Hz, 1H, −CH=CHAr)
9a	OCH_3	$(CH_2)_3OPO(OH)_2$	2.04 (m, 2H, −CH_2−), 3.10 (t, J = 2.2 Hz, 1H, HC≡C−), 3.82 (s, 3H, N7−CH_3), 3.89−3.95 (m, 2H, N3−CH_2−), 4.04 (s, 3H, OCH_3), 4.09−4.15 (m, 2H, O−CH_2−), 4.60 (d, J = 2.6 Hz, 2H, −CH_2−N1), 6.91−7.70 (m, 6H, $4H_{arom}$, $2H_{vinylic}$)
9b[c]	OCH_3	$(CH_2)_3OPO(ONa)_2$	1.81 (m, 2H, −CH_2−), 2.38 (t, J = 2.2 Hz, 1H, HC≡C−), 3.49 (s, 3H, N7−CH_3), 3.51 (s, 3H, OCH_3), 3.77 (m, 2H, N3−CH_2−), 3.99 (m, 2H, O−CH_2−), 4.42 (d, J = 2.6 Hz, 2H, −CH_2−N1), 6.51−7.10 (m, 6H, $4H_{arom}$, $2H_{vinylic}$)
10a	$OP(OH)_2$	CH_3	3.10 (t, J = 2.3 Hz, 1H, HC≡C−), 3.48 (s, 3H, N3−CH_3), 4.03 (s, 3H, N7−CH_3), 4.24 (br s, 2H, 2 POH), 4.59 (d, J = 2.3 Hz, 2H, −CH_2−N1), 7.19−7.70 (m, 6H, styryl)
10b[c]	$OP(ONa)_2$	CH_3	2.55 (t, J = 2.2 Hz, 1H, HC≡C−), 3.17 (s, 3H, N3−CH_3), 3.49 (s, 3H, N7−CH_3), 4.32 (s, 2H, −CH_2−N1), 6.39−6.91 (m, 6H, $4H_{arom}$, $2H_{vinylic}$)

[a] In DMSO-d_6 unless otherwise indicated. [b] $CDCl_3$. [c] D_2O.

Table 3. Selected ^{13}C and ^{31}P NMR Data of Phosphate Prodrugs



compd	R^1	R^2	^{13}C NMR δ (ppm)	^{31}P NMR δ (ppm)
8[a]	OCH_3	$(CH_2)_3OPO(O^tBu)_2$	29.75 (d, J = 7.6 Hz, −CH_2−CH_2−O−P), 30.75 (d, J = 4.6 Hz, $(CH_3)_3$C−O−P), 31.26 (N1−CH_2−), 32.41 (N7−CH_3), 41.66 (N3−CH_2−), 56.22 (O−CH_3), 65.45 (d, J = 6.1 Hz, −CH_2−O−P), 71.23 (HC≡C−CH_2−), 83.10 (d, J = 7.6 Hz, $(CH_3)_3C$−O−P), 95.12 (HC≡C−CH_2−), 108.73 (C5), 137.75 (C8), 149.39 (C4), 151.34 (C2), 160.92 (C6), 112.25, 113.61, 116.14, 120.77, 130.78, 139.60 (styryl)	−9.57 (s)
9a[b]	OCH_3	$(CH_2)_3OPO(OH)_2$	28.51 (d, J = 7.6 Hz, −CH_2−CH_2−O−P), 29.81 (N1−CH_2−), 31.39 (N7−CH_3), 55.10 (O−CH_3), 63.05 (d, J = 6.1 Hz, −CH_2−O−P), 72.63 (HC≡C−CH_2−), 79.51 (HC≡C−CH_2−), 107.10 (C5), 137.00 (C8), 147.80 (C4), 149.92 (C4), 159.50 (C6), 112.38, 112.83, 115.11, 120.14, 129.66, 136.76, 149.92 (styryl)	−1.07 (s)
9b[c]	OCH_3	$(CH_2)_3OPO(ONa)_2$	nd[d]	+0.81 (s)
10a[b]	$OP(OH)_2$	CH_3	29.36 (N3−CH_3), 29.81 (N1−CH_2−), 31.36 (N7−CH_3), 72.60 (HC≡C−CH_2−), 79.51 (HC≡C−CH_2−), 107.04 (C5), 136.06 (C8), 148.16 (C4), 150.01 (C4), 153.07 (C6), 113.29, 118.72, 120.75, 123.24, 129.69, 136.88, 149.67, 151.95 (d, J = 6.1 Hz, C3′−O−P), (styryl)	−6.04 (s)
10b[c]	$OP(ONa)_2$	CH_3	nd	−4.07 (s)

[a] In $CDCl_3$. [b] In DMSO-d_6. [c] In D_2O. [d] nd, not determined.

curve of **9b** was shifted to the left (Figure 2). It is likely that the rat brain striatal membrane preparation used for the A_{2A} binding studies contained phosphatase activity, and the relatively high A_{2A} affinity of MSX-3 may be − at least partly − due to degradation to **5e** by phosphatases present in the preparation during the 30-min incubation period. Preincubation with alkaline phosphatase led to virtually quantitative hydrolysis of the phosphoric acid ester yielding **5e**, and therefore, the binding curve was shifted to the left.

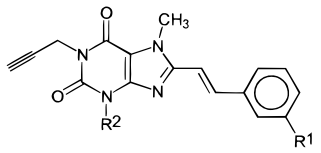
Similar studies with phosphate prodrug **9b** at rat A_1 AR were performed. Compound **9b** showed identical apparent K_i values (K_i = 1.12 ± 0.35 μM, n = 5) at rat A_1 AR without and after preincubation with alkaline phosphatase. The K_i value was similar to that obtained for **5e** (Table 4). It appears that the presence of phosphatase activity in the rat brain cortical membrane preparations may already lead to complete hydrolysis under the assay conditions (90 min of incubation), and therefore preincubation with phosphatase shows no effect. In contrast to the A_1 AR assay, incubation using a striatal membrane preparation for the A_{2A} AR assay only lasts for 30 min. Hydrolysis by endogenous phosphatases is not complete during the A_{2A} assay procedure. The binding studies with prodrug **9b** confirm that the compound is rapidly degraded to **5e** by phosphatase and that **9b** itself will not interact with A_1 AR in low concentrations.

In conclusion, we have developed a highly water-soluble phosphate prodrug of a potent, A_{2A}-selective AR antagonist, which may be a valuable pharmacological tool for studying A_{2A} receptors in vivo and, in fact, has already proven its usefulness in recent studies.[4]

Experimental Section

Chemical Synthesis. ^{1}H and ^{13}C NMR spectra were performed on a Bruker AC-200 spectrometer. The chemical shifts of the remaining protons of the deuterated solvents served

Table 4. AR Affinity and Selectivity of 1-Propargyl-8-styrylxanthine Derivatives Bearing Functional Groups for Prodrug Formation



			$K_i \pm$ SEM (μM)				
compd	R^1	R^2	A_1 affinity rat brain cortical membranes [^{3}H]CHA	A_{2A} affinity rat brain striatal membranes [^{3}H]CGS21680	A_{2B} cyclase activity human recombinant receptor in CHO cell membranes, inhib of NECA-stimulated AC	A_3 affinity human recombinant receptor in CHO cell membranes [^{3}H]NECA	selectivity A_{2A} vs A_1 (A_1/A_{2A})
11 (BS-DMPX)	Br	CH_3	1.2[11]	0.0082[11]	>10[12]	>10[12]	146
5b (MS-DMPX)	OCH_3	CH_3	1.28 ± 0.65	0.012 ± 0.005	nd[a]	nd	106
5c	OCH_3	$(CH_2)_2OH$	1.04 ± 0.04	0.039 ± 0.003	nd	nd	27
5d	Br	$(CH_2)_2OH$	1.28 ± 0.14	0.025 ± 0.010	nd	nd	52
5e (MSX-2)	OCH_3	$(CH_2)_3OH$	0.900 ± 0.010	0.008 ± 0.003	>10	>10	113 (rat)
			2.50 (1.70−3.60)[b]	(0.005 ± 0.0006)[c] (0.015 ± 0.002)[d]			500 (humans)
5f	Br	$(CH_2)_3OH$	0.625 ± 0.065	0.013 ± 0.003	nd	nd	48
6	NH_2	CH_3	1.07 ± 0.01	0.042 ± 0.001	nd	nd	25
7	OH	CH_3	0.940 ± 0.023	0.021 ± 0.006	nd	nd	45

[a] nd, not determined. [b] K_i (95% confidence limits) from [^{3}H]CCPA binding at human recombinant A_1 AR. [c] $K_i \pm$ SEM from [^{3}H]CGS21680 binding at human recombinant A_{2A} AR. [d] $K_i \pm$ SEM from [^{3}H]CGS21680 binding at human post-mortem brain (caput nuclei caudati) A_{2A} AR.

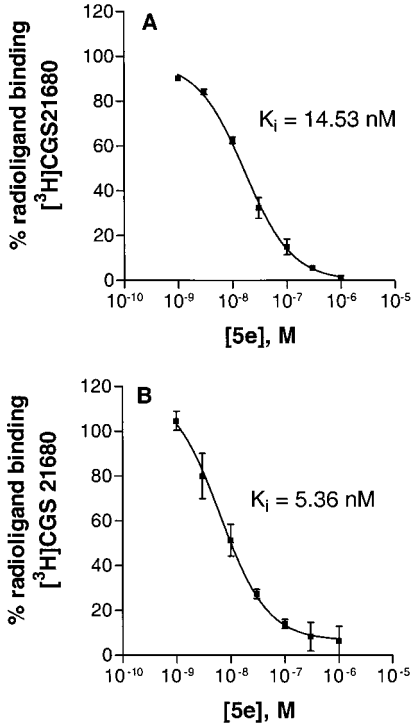


Figure 1. Binding of **5e** (MSX-2) to human A_{2A} AR: A, human post-mortem brain (membrane preparation from caput nuclei caudati); B, human recombinant A_{2A} AR expressed in CHO cells (membrane preparation). Data points represent means ± SEM of three independent experiments (in triplicate).

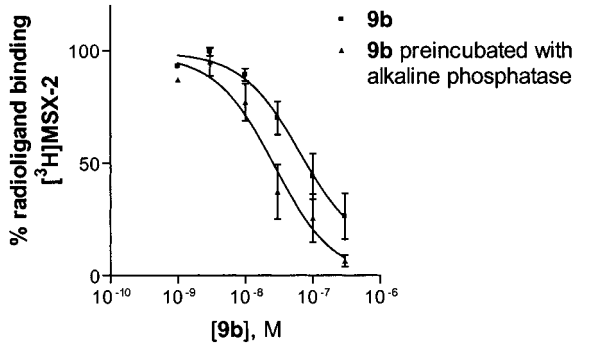


Figure 2. Binding of phosphate prodrug **9b** (MSX-3) to A_{2A} adenosine receptors of rat brain membranes without and after preincubation with alkaline phosphatase ($n = 6$).

as internal standards; for spectra recorded in DMSO-d_6: δ ^{1}H 2.51, δ ^{13}C 39.7 and in D_2O: δ ^{1}H 4.65. Tetramethylsilane was used as internal standard for spectra recorded in $CDCl_3$. ^{31}P NMR spectra were recorded on a Bruker AMX-400 spectrometer; orthophosphoric acid (85%) was used as an external standard. All compounds were checked for purity by TLC on silica gel 60 F_{254} (Merck), using dichloromethane:methanol (9:1) and 2-propanol:NH_3:H_2O (6:3:1) as the mobile phase, respectively. Melting points were taken on a Büchi 510 melting point apparatus and are uncorrected. Elemental analyses were performed by the Institute of Inorganic Chemistry, University of Würzburg.

5,6-Diamino-3-propargyluracil (1) was prepared from 6-aminouracil via regioselective alkylation followed by nitrosation and reduction as described.[20,25] **6-Amino-5-(3-bromocinnamoylamino)-3-propargyluracil (2c)** was prepared as described.[11] Alkaline phosphatase (AP) from SIGMA (P-5521, 3.3 mg protein/mL, 2300 units/mg protein) was used for the enzymatic hydrolysis of **9b**.

6-Amino-5-(3-nitrocinnamoylamino)-3-propargyluracil (2a) and 6-Amino-5-(*m*-methoxycinnamoylamino)-3-propargyluracil (2b): General Procedure. A suspension of **1** (360 mg, 2.0 mmol), (*E*)-3-nitrocinnamic acid or (*E*)-3-methoxycinnamic acid (2.2 mmol), respectively, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (420 mg, 2.2 mmol) in methanol (15 mL) was stirred at room temperature for 4 h. The formed precipitate was collected by filtration and washed with methanol (15 mL) and subsequently with H_2O (15 mL) to afford compound **2a** or **2b**.

6-Amino-1-methyl-5-(*m*-nitrocinnamoylamino)-3-propargyluracil (3a) and 6-Amino-1-methyl-5-(*m*-methoxycinnamoylamino)-3-propargyluracil (3b): General Procedure. To a solution of **2a** or **2b** (3.0 mmol) in DMF (30 mL) were added K_2CO_3 (500 mg, 3.6 mmol) and CH_3I (1.88 mL, 30 mmol). The mixture was stirred at room temperature for 16 h and the product was precipitated by the addition of H_2O (100 mL), collected by filtration, and washed with H_2O (20 mL) and

subsequently with methanol (20 mL). Further purification was achieved by dissolution in DMF (10 mL) and precipitation by the addition of H_2O (50 mL).

6-Amino-1-(2-hydroxyethyl)-5-(*m*-methoxycinnamoylamino)-3-propargyluracil (3c), 6-Amino-1-(2-hydroxyethyl)-5-(*m*-bromocinnamoylamino)-3-propargyluracil (3d),6-Amino-1-(3-hydroxypropyl)-5-(*m*-methoxycinnamoylamino)-3-propargyluracil (3e), and 6-Amino-1-(3-hydroxypropyl)-5-(*m*-bromocinnamoylamino)-3-propargyluracil (3f): General Procedure. To a solution of compound **2b** or **2c** (3.0 mmol) in DMF (30 mL) were added K_2CO_3 (500 mg, 3.6 mmol) and 2-hydroxyethyl bromide or 3-hydroxypropyl bromide (60 mmol), respectively. The mixture was stirred at 60 °C for 24 h and DMF was removed in vacuo. The residue was dissolved in methanol (10 mL) and the product was precipitated by the addition of H_2O (40 mL). The precipitate was collected by filtration and washed with a mixture of methanol and H_2O (1:1, 20 mL).

3-Methyl-8-(*m*-nitrostyryl)-1-propargylxanthine (4a), 8-(*m*-Methoxystyryl)-3-methyl-1-propargylxanthine (4b), 3-(2-Hydroxyethyl)-8-(*m*-methoxystyryl)-1-propargylxanthine (4c), 8-(*m*-Bromostyryl)-3-(2-hydroxyethyl)-1-propargylxanthine(4d),3-(3-Hydroxypropyl)-8-(*m*-methoxystyryl)-1-propargylxanthine (4e), and 8-(*m*-Bromostyryl)-3-(3-hydroxypropyl)-1-propargylxanthine (4f): General Procedure (Method A). Compounds **3 (3a−3f)** (3.0 mmol) were dissolved in a mixture of MeOH (100 mL) and 20% aq NaOH solution (20 mL) and heated at 50−60 °C for 2−3 h. After cooling, the solution was acidified to pH 4 by the addition of concd HCl, and the formed precipitate was collected by filtration and washed with H_2O. Purification was achieved by dissolution in DMF (10 mL) at ca. 80 °C and precipitation by the addition of H_2O (50 mL).

3-(3-Hydroxypropyl)-8-(*m*-methoxystyryl)-1-propargylxanthine (4e) (Method B). A suspension of **3e** (150 mg, 0.38 mmol) and catalytic amounts of $(NH_4)_2SO_4$ in 15 mL of hexamethyldisilazane was heated at 110−120 °C for 24 h. The solvent was removed in vacuo. The residue was cooled to 0 °C and hydrolyzed by the addition of a mixture of 10 mL of methanol and 10 mL of water. The precipitate was filtered off and washed with 20 mL of methanol and subsequently with 50 mL of water. Further purification was performed as described above (method A).

3,7-Dimethyl-8-(*m*-nitrostyryl)-1-propargylxanthine (5a), 3,7-Dimethyl-8-(*m*-methoxystyryl)-1-propargylxanthine (5b), 3-(2-Hydroxyethyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargylxanthine (5c), 8-(*m*-Bromostyryl)-3-(2-hydroxyethyl)-7-methyl-1-propargylxanthine (5d), 3-(3-Hydroxypropyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargyl-xanthine (5e), and 8-(*m*-Bromostyryl)-3-(3-hydroxypropyl)-7-methyl-1-propargylxanthine(5f): General Procedure. Compounds **4 (4a−4f)** (2.3 mmol) were dissolved in DMF (20 mL); K_2CO_3 (640 mg, 4.6 mmol) and MeI (0.19 mL, 3.0 mmol) were added. The mixture was stirred at room temperature overnight. Then H_2O (80 mL) was added to precipitate the product, which was collected by filtration. The product was treated with 10% aq NaOH solution (15 mL) to dissolve impurities. Further purification was achieved by dissolution in DMF (10 mL) at ca. 80 °C and precipitation by the addition of H_2O (50 mL).

8-(*m*-Aminostyryl)-3,7-dimethyl-1-propargylxanthine (6). To a stirred solution of **5a** (0.37 g, 1 mmol) in a mixture of acetic acid (100 mL) and H_2O (30 mL) was added zinc powder (0.65 g, 10 mmol) in small portions. The mixture was stirred for 1.5 h and filtered, and the filtrate was evaporated to dryness. The residue was treated with H_2O (50 mL) and neutralized with 2 N NH_4OH solution. The suspension was extracted three times with 100 mL of CH_2Cl_2. The pooled organic extracts were dried with Na_2SO_4 and evaporated. The solid residue was chromatographed on silica gel (CH_2Cl_2:MeOH = 99.5:0.5) to give pure compound **6**.

3,7-Dimethyl-8-(*m*-hydroxystyryl)-1-propargylxanthine (7). To a stirred solution of **5b** (1.40 g, 4.0 mmol) in CH_2Cl_2 (200 mL) was added BBr_3 (1.89 mL, 20.0 mmol) slowly at 0 °C. The reaction mixture was stirred at room temperature for 3 h and then poured into an ice-cooled saturated $NaHCO_3$ solution (250 mL). The precipitated product was filtered off and recrystallized from MeOH:CH_2Cl_2 (2:1, 50 mL) to yield **7**.

Di-*O*-*tert*-butyl 3-*O*-Hydroxypropyl-8-(*m*-methoxystyryl)-7-methyl-1-propargylxanthine Phosphate Triester (8). Method A. Compound **5e** (100 mg, 0.25 mmol) was dissolved in a mixture of 1*H*-tetrazole (210 mg, 3.0 mmol) in 10 mL of dry acetonitrile and 10 mL of dry CH_2Cl_2. At room temperature di-*tert*-butyl *N*,*N*-diethylphosphoramidite (313 mg, 1.25 mmol) was added under nitrogen and the mixture was stirred for 2 h. Then 70% *tert*-butyl hydroperoxide solution (5 mL, 35 mmol) was added and the mixture was stirred for 1 h. At 0 °C 15 mL of 10% $NaHSO_3$ solution was added and the mixture was stirred for 15 min more. Then the mixture was extracted three times with 10 mL of CH_2Cl_2, and the organic layer was washed with water. Evaporation of solvent yielded a yellow oil, which was purified by column chromatography (CH_2Cl_2:MeOH = 98:2). Fractions containing **8** were combined and concentrated in vacuo. The product could be crystallized by the addition of hexane (5 mL).

3-(3-*O*-Phosphatylpropyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargylxanthine (9a). Method A. Compound **8** (100 mg, 0.17 mmol) was dissolved in CH_2Cl_2 (10 mL). At room temperature 0.3 mL of 99% trifluoroacetic acid (3.9 mmol) was added and the solution was stirred for 1.5 h. The mixture was evaporated in vacuo and the residue was coevaporated with water and subsequently washed with water to give **9a**.

Method B. Compound **5e** (500 mg, 1.27 mmol) was suspended in 20 mL of dry trimethyl phosphate. Under nitrogen phosphorus oxychloride (0.2 mL, 2.0 mmol) was added and the mixture was stirred at room temperature for 2 h. The product was separated through the addition of 20 mL of H_2O. The precipitate was filtered off and washed with 20 mL of H_2O and subsequently with 20 mL of methanol. The product was treated with 50 mL of $CHCl_3$ at 60 °C to dissolve impurities and collected by filtration to yield **9a**.

3,7-Dimethyl-8-(*m*-*O*-phosphatylstyryl)-1-propargylxanthine (10a). Compound **7** (90 mg, 0.27 mmol) was dissolved in a mixture of 1*H*-tetrazole (210 mg, 3.0 mmol) dissolved in 10 mL of dry acetonitrile and 20 mL of dry THF. At room temperature di-*tert*-butyl *N*,*N*-diethylphosphoramidite (313 mg, 1.25 mmol) was added under nitrogen and the mixture was stirred for 2 h. Then 70% *tert*-butyl hydroperoxide solution (5 mL, 35 mmol) was added, and the mixture was stirred for 1 h. At 0 °C 15 mL of 5% $NaHSO_3$ solution was added and the mixture was stirred for 15 min more. Then the mixture was extracted three times with 15 mL of CH_2Cl_2 and the organic layer was washed with water. After removal of the solvent, the residue was dissolved in 20 mL of CH_2Cl_2 and insoluble impurities were filtered off. Trifluoroacetic acid (0.2 mL, 2.6 mmol) was added at room temperature and the solution was stirred for 12 h. A precipitate was formed, which was filtered off and washed with 15 mL of CH_2Cl_2. The residue was recrystallized from water to give **10a**.

8-(*m*-Methoxystyryl)-7-methyl-3-(3-*O*-phosphatylpropyl)-1-propargylxanthine Disodium Salt (9b) and 3,7-Dimethyl-8-(*m*-*O*-phosphatylstyryl)-1-propargylxanthine Disodium Salt (10b): General Procedure. A suspension of compound **9a** or **10a** (0.25 mmol) and 25 g of ion-exchange resin (Dowex WX4, Na^+ form) in 250 mL of H_2O was gently stirred at room temperature for 24 h. The resin was filtered off, and the solution was concentrated in vacuo and freeze-dried. The product could be purified by size exclusion chromatography using Biorad P2-gel and water as eluent. The different fractions were checked by TLC, and fractions containing product were collected, frozen, and lyophilized to give **9b** or **10b** as colorless powders.

Determination of Water Solubility of 9b (MSX-3). A suspension of **9b** (MSX-3) in water was prepared and stirred at room temperature for 24 h. After centrifugation (1 h, 10 000 rpm) and subsequent filtration through a micropore filter (0.2 μm), a saturated solution was obtained. This solution was diluted with water (1:99), and the UV absorption was deter-

mined at $\lambda_{max} = 282$ nm. The concentration of the solution was calculated using a standard calibration curve (five single concentrations from 10−100 μM, $\epsilon = 11\,800$ L·cm^{-1}·mol^{-1}). Results are mean values from three separate experiments. The water solubility of **9b** (MSX-3) was determined to be 9 mg/mL (17 mM).

Radioligand Binding Assays. The compounds were tested in radioligand binding assays for affinity to A_1 and A_{2A} ARs in rat cortical membrane and rat striatal membrane preparations, respectively. The A_1-selective agonist [^{3}H]-N^6-cyclohexyladenosine (CHA; 1 nM) was used as A_1 ligand and the A_{2A}-selective agonist [^{3}H]-2-[[[4-(carboxyethyl)phenyl]ethyl]amino]-5′-(*N*-ethylcarbonylamino)adenosine (CGS21680; 5 nM) as A_{2A} ligand as previously described.[26−28] Inhibition of receptor−radioligand binding was determined by a range of five to six concentrations of the compounds in triplicate in at least three separate experiments. The Cheng−Prusoff equation and K_D values of 1 nM for [^{3}H]CHA and 14 nM for [^{3}H]CGS21680 were used to calculate the K_i values from IC_{50} values, determined by the nonlinear curve-fitting program GraphPad Prism, version 2.0 (GraphPad, San Diego, CA).

A_1 and A_3 AR affinities to human recombinant receptors expressed in CHO cell membranes were determined as described using [^{3}H]-2-chloro-N^6-cyclopentyladenosine (CCPA) as A_1, [^{3}H]CGS21680 as A_{2A}, and [^{3}H]-5′-(*N*-ethylcarbonylamino)-adenosine (NECA) as A_3 radioligands.[29] A_{2A} AR binding to human recombinant cells expressed in CHO cells was performed analogously using 5 nM [^{3}H]CGS21680.[29] The following K_d values were used to calculate K_i values: [^{3}H]CCPA, A_1 AR, 0.6 nM; [^{3}H]CGS21680, A_{2A} AR, 32 nM; [^{3}H]NECA, A_3 AR, 6 nM.

Preparation of Post-Mortem Brain (caput nuclei caudati) Membrane Preparation. The brains of three people (2 female, 1 male, ages: 84, 71, and 73), who had no history of CNS disorders ("control brains"), were dissected 12−54 h post-mortem and stored at −80 °C. Crude membranes for radioligand binding experiments were prepared as follows: Frozen caput nuclei caudati were thawed and cut into smaller pieces. The tissue from the three brains was pooled and homogenized on ice (polytron, 10 s with high speed) in ice-cold HEPES buffer (consisting of 15 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4, freshly prepared). Then the homogenate was centrifuged for 20 min at 4 °C, 40000*g*. The membrane pellet was resuspended in HEPES, washed twice, and then stored in aliquots at −80 °C with a protein concentration of 7.1 mg/mL. Just before the experiment the tissue was washed once again with TRIS buffer (pH 7.4, 50 mM) for 20 min at 4 °C and 40000*g*.

Radioligand Binding Assays at Human Post-Mortem Brain A_{2A} ARs. Radioligand binding assays were performed essentially as described.[28] ^{3}[H]CGS21680 was used as A_{2A} ligand in a concentration of 5 nM. The protein concentration in the assays was 50 μg/mL. A K_d value of 22 nM[30] was used to calculate K_i values.

The nonspecific binding amounted to ca. 40% of total binding, while it was somewhat higher (ca. 50%) in assays using recombinant A_{2A} receptors.

Adenylate Cyclase Assays. Inhibition of NECA-induced stimulation of adenylate cyclase by **5e** was determined at human recombinant A_{2B} receptors as described.[29]

Effects of Phosphatase on AR Binding of Phosphate Prodrug 9b (MSX-3). For these experiments, [^{3}H]CCPA[31] (0.5 nM) was used as A_1-selective radioligand and [^{3}H]-3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-1-propargylxanthine([^{3}H]-MSX-2, 1 nM) as A_{2A}-selective radioligand.[1,31] Binding assays were performed essentially as described.[32,33]

A 0.1 mM solution of **9b** in TRIS-HCl buffer (pH 7.4) was incubated at 37 °C, alkaline phosphatase (AP) (3.5 μg protein/mL) was added, and the solution was gently shaken while incubated for 1 h. In a separate experiment it was confirmed that **9b** hydrolyzed quantitatively under these conditions as detected by TLC with UV detection (eluent: 2-propanol:NH_3:H_2O = 6:3:1). Aliquots from the incubated solution were taken and diluted with TRIS-HCl buffer to the final concentrations of 0.003, 0.01, 0.03, 0.1, 0.3, and 1 μM. The same dilutions were prepared for a solution of **9b** which had not been pretreated with AP. The K_i values were determined as described above.

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

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