Novel Amino Acid Derived Natural Products from the Ascidian *Atriolum robustum*: Identification and Pharmacological Characterization of a Unique Adenosine Derivative

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Received November 5, 2003

Investigation of the methanolic extract of the Australian ascidian *Atriolum robustum* led to the isolation and characterization of five new amino acid derived structures (**1**−**5**). The structures were elucidated employing spectroscopic techniques (NMR, MS, UV, and IR). The absolute stereochemistry of **1** and **2** was established by chemical degradation, derivatization, and chiral GC−MS analysis. Structures **4** and **5** are complex nucleosides containing rare methylthioadenosine and methylsulfinyladenosine moieties, respectively. In radioligand binding studies the 5′-deoxy-5′-methylthioadenosine-2′,3′-diester **4** exhibited affinity for A_1 and A_3 adenosine receptors with K_i values below 10 μM. Its affinity was somewhat lower for A_{2A} ($K_i = 17$ μM) and much lower for A_{2B} adenosine receptors. Analytical experiments using capillary electrophoresis showed that compound **4** was stable under the conditions of radioligand binding studies. Incubation with carboxylesterase resulted in slow hydrolysis of the adenosine derivative to 5′-deoxy-5′-methylthioadenosine (MTA), which was about 10-fold more potent at adenosine receptors than compound **4**. Thus, the 2′,3′-diester derivative **4** may act as a lipophilic prodrug of MTA in addition to its own adenosine receptor activity. GTP shift experiments indicated that the adenosine derivative was a partial agonist at A_1 adenosine receptors of rat brain cortical membranes. Compound **4** inhibited cAMP accumulation in Chinese hamster ovary (CHO) cell membranes recombinantly expressing the human A_3 adenosine receptor, thus indicating that the adenosine derivative also acted as a partial agonist at A_3ARs. Homology models of the A_1 and the A_3 adenosine receptors in their putative active and inactive conformations were built and used for docking of the sterically demanding compound **4**. It was found that this ligand fit well into the binding pockets of both receptor subtypes because of its highly flexible structure, although in somewhat different binding modes.

Introduction

Marine ascidians are known to be a rich source of chemically diverse secondary metabolites with often remarkable biological activities.[1,2] In many cases these compounds are simple amino acid derivatives[3] or more complex alkaloids.[4] In particular, ascidians of the family Didemnidae have been investigated intensively because of their unusual, cytotoxic metabolites.[5,6] As part of our continuing interest in ascidian chemistry, our attention focused on the Australian ascidian *Atriolum robustum* Kott, 1983 (Didemnidae). The ascidian was collected at Heron Islands, Wistari Reef, the Great Barrier Reef, Australia. We report here the isolation of three new amino acid derived compounds (**1**−**3**) and two new, more complex nucleosides containing the rare methylthioadenosine[7] (**4**) and methylsulfinyladenosine[8] (**5**) moieties. The new compounds **1**, **4**, and **5** contain a unique 3-(4-hydroxy-phenyl)-2-methoxyacrylic acid moiety, to date only described from an ascidian of the genus *Botryllus*.[9] Deamination of tyrosine catalyzed by tyrosine ammonia-lyase (TAL)[10] and subsequent methoxylation may have led to this moiety. Compounds **2**−**5** all contain a urocanic acid moiety[11] probably generated by an enzyme-catalyzed deamination (histidine ammonia-lyase, HAL) of histidine.[12]

The present study explores the binding affinities of the major novel adenosine derivative **4** at the different adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) because of its structural relation to the physiological agonist adenosine. Adenosine receptors (ARs) belong to the class of G-protein-coupled receptors (GPCRs) and may be coupled to adenylate cyclase (AC) as a second messenger system. A_1 and A_3 AR stimulation leads to an inhibition of the enzyme, while A_{2A} and A_{2B} AR activation causes a stimulation of AC.[13] Compound **4** contains 5′-deoxy-5′-methylthioadenosine (MTA) with the ribose moiety being esterified with 3-(4-hydroxyphenyl)-2-methoxyacrylic acid at the 2′-position and with urocanic acid at the 3′-position. As previously reported, the ribose moiety appears to be required for both high affinity and intrinsic activity for this class of compounds.[14] MTA had previously been described as an agonist[15] or a partial agonist[16] at A_1ARs, a partial

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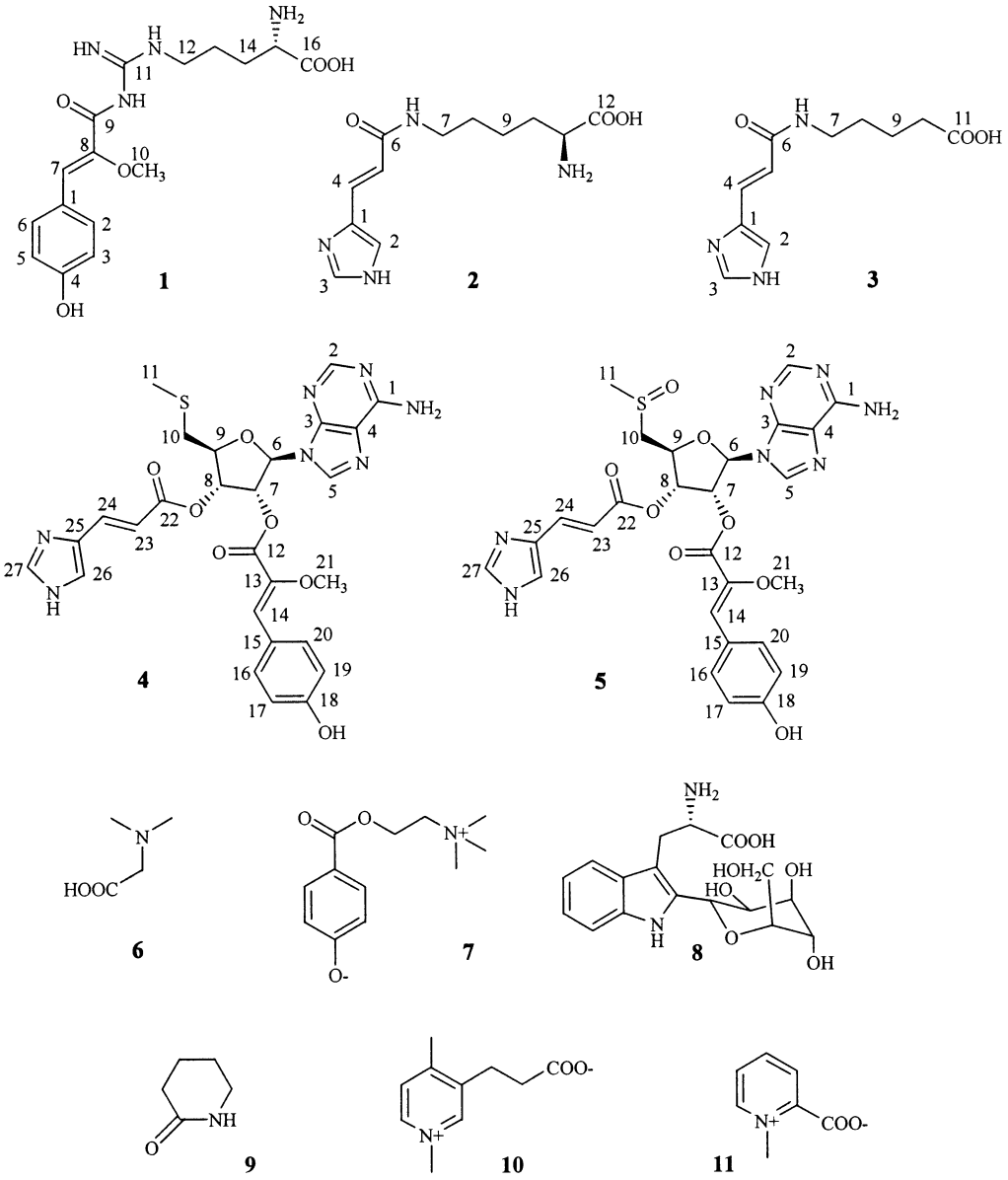
10.1021/jm031092g CCC: $27.50
Published on Web 03/30/2004

Scheme 1



agonist at A_{2A}ARs,[17] and an antagonist[15,18] or a weak partial agonist[17] at A_{2B}ARs. The potency of MTA at A_3ARs has not been reported to our knowledge. The present study compares the binding affinities and intrinsic activities of the new natural product **4** with those of the related nucleoside MTA. In addition, the chemical and enzymatic stability of compound **4** was investigated.

Furthermore, receptor docking experiments were performed with the nucleoside derivative **4**. Since no X-ray structures of the ARs were available, homology modeling was applied on the basis of the crystal structure of bovine rhodopsin.[19] This GPCR is integral to vision; it contains a retinal chromophore that is covalently linked to lysine-296 (Lys296). Upon absorption of a photon, the inverse agonist 11-*cis*-retinal isomerizes to *all-trans*-retinal.[20] Although there are some indications of the resulting conformational changes in the receptor,[21−25] the structure of metarhodopsin II (signaling state) is still unknown. On the basis of the hypothesis that the main differences in the shape of the binding pocket result from changes in the side chain torsion of tryptophan 247 (Trp247), we built models of the active conformations of the A_1 and the A_3 adenosine receptor and used them in our docking experiments. Thus, the pharmacological results characterizing the new natural product **4** as a partial agonist at A_1 and A_3 ARs could be rationalized by modeling complexes of **4** with the receptor proteins.

Results and Discussion

Chemistry. Mass spectral analysis of compound **1** indicated it to have the molecular formula $C_{16}H_{22}N_4O_5$ and thus eight degrees of unsaturation. Its ^{13}C NMR data contained a total of 16 resonances for one methyl, three methylene, and six methine groups and six quarternary carbons. These data also revealed the presence of seven double bonds (4 × CC; 1 × CN; 2 × CO). One degree of unsaturation could be assigned to the presence of a 1,4-disubstituted benzene ring because of characteristic resonances (δ 6.84, d, J = 8.5 Hz, 2H; δ 7.56, d, J = 8.5 Hz, 2H; see Table 1) observed in the ^1H NMR spectrum of **1**. After assignment of all protons to their directly bonded carbon atoms via a $^1H-^{13}C$ 2D

Table 1. ^{1}H NMR Spectral Data for Compounds **1**−**3** (δ in ppm, J in Hz)[a]

carbon	**1**[b]	**2**[b]	**3**[b]
1			
2	7.56 (1H, d, J = 8.5)	7.36 (1H, brs)	7.46 (1H, brs)
3	6.84 (1H, d, J = 8.5)	8.04 (1H, brs)	8.25 (1H, brs)
4		7.18 (1H, d, J = 15.7)	7.23 (1H, d, J = 15.7)
5	6.84 (1H, d, J = 8.5)	6.35 (1H, d, J = 15.7)	6.42 (1H, d, J = 15.7)
6	7.56 (1H, d, J = 8.5)		
7	6.78 (1H, s)	3.21 (2H, t, J = 6.3)	3.16 (2H, t, J = 6.0)
8		1.52 (2H, m)	1.45 (2H, m)
9		1.34 (2H, m)	1.45 (2H, m)
10	3.59 (3H, s)	1.80 (2H, m)	2.13 (2H, t, J = 6.4)
11		3.65 (1H, t, J = 6.1)	
12	3.12 (2H, t, J = 6.4)		
13	1.54 (2H, m)		
14	1.71 (1H, m); 1.84 (1H, m)		
15	4.23 (1H, t, J = 6.4)		
16			

[a] All assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [b] D_2O + CD_3OD, 300 MHz.

NMR shift correlated measurement (heteronuclear single quantum coherence, HSQC), it was possible to deduce a second fragment of the molecule from the results of a ^{1}H−^{1}H correlation spectroscopy (COSY) measurement. Thus, analysis of the correlation spectrum of **1** gave evidence for connectivities from CH_2-12 to CH-15. Characteristic downfield-shifted ^{1}H and ^{13}C NMR resonances for both CH_2-12 and CH-15 (δ 3.12 and 41.6; δ 4.23 and 55.6) indicated C-12 and C-15 to be α to nitrogen. A characteristic ^{13}C NMR chemical shift for carbon C-11 (δ 157.6) and the long-range CH couplings (heteronuclear multiple-bond correlation, HMBC) observed between C-16 (δ 179.2) and H-15 and between C-11 and H_2-12 were evident for an arginine moiety. The chemical shift of C-4 (δ 157.7) showed the aromatic ring to be para-hydroxylated. The double bond Δ^7 has to be connected to C-1 and thus to the 1,4-disubstituted benzene ring because of ^{1}H−^{13}C HMBC couplings observed between C-2 and H-7 and between C-6 and H-7. The chemical shift of C-7 (δ 122.0) established the geometry of double bond Δ^7 to be *Z*.[9] The linkage between C-8 and the carbonyl carbon C-9 (δ 166.8) and between C-8 and the methoxyl group (C-10) was proven by long-range CH correlations between C-9 and H-7 and between C-8 and H_3-10. The final connection between the arginine moiety and C-9, and hence the planar structure of **1**, followed by deduction.

Acid hydrolysis followed by amino acid analysis using chiral GC−MS enabled the absolute stereochemistry of **1** to be deduced. The L-configuration for the amino acid residue arginine was established by comparative GC analysis of its pentafluoropropyl isopropyl ester derivative with the correspondingly derivatized standard D- and L-arginine (see Experimental Section).

Compound **2** was obtained as a white solid. The molecular formula $C_{12}H_{18}N_4O_3$, established by high-resolution fast atom bombardment mass spectometry (HRFABMS), showed six degrees of unsaturation. ^{1}H NMR resonances observed at δ 6.35 (d, J = 15.7 Hz) and δ 7.18 (d, J = 15.7 Hz) suggested the presence of a trans double bond, and two singlets at δ 8.04 and δ 7.36 were indicative of a heterocycle. The ^{13}C NMR data, in conjunction with HMBC couplings observed between C-1 and H-2, H-3, H-4, and H-5, between C-2 and both H-3 and H-4, and between C-6 and both H-4 and H-5 were in good agreement with the presence of a urocanic acid moiety.[26] From the ^{1}H−^{1}H COSY spectrum, the C-7 to C-11 part of the molecule was assigned. Downfield shifts of the resonances for H_2-7 (δ 3.21) and H-11 (δ 3.65) together with a long-range CH coupling observed between carbonyl C-12 and H-11 clearly delineated this part of the molecule to be a lysine moiety. The amidic bond between the lysine moiety and the urocanic acid moiety was established because of an HMBC coupling observed between C-6 and H_2-7. Chiral GC−MS showed the amino acid lysine to have L-configuration.

Table 2. ^{13}C NMR Spectral Data for Compounds **1**−**3**

	1[a,b]		**2**[a,b]		**3**[a,b]	
carbon	δ_C (mult)	HMBC[d]	δ_C (mult)	HMBC[d]	δ_C (mult)	HMBC[d]
1	126.0 (s)[c]	3, 5	133.2 (s)	2, 3, 4, 5	132.2 (s)	2, 3, 4, 5
2	132.9 (d)	7	121.7 (d)	3, 4	122.1 (d)	3, 4
3	116.7 (d)		137.6 (d)	2	137.1 (d)	2
4	157.7 (s)	2, 6	129.4 (d)	5	128.2 (d)	5
5	116.7 (d)		121.2 (d)	4	121.3 (d)	4
6	132.9 (d)	7	168.9 (s)	4, 5, 7	168.6 (s)	5, 7
7	122.0 (d)	2, 6	40.2 (t)	8, 9	40.3 (t)	
8	146.9 (s)	7, 10	29.1 (t)	7, 9	29.0 (t)	7, 9, 10
9	166.8 (s)	7	22.9 (t)	7, 8, 11	23.7 (t)	7, 8, 10
10	60.3 (q)		31.1 (t)	8, 11	37.0 (t)	
11	157.6 (s)	12	55.7 (d)	9, 10	182.9 (s)	10
12	41.6 (t)	13	175.6 (s)	11		
13	25.4 (t)	14				
14	29.9 (t)	12				
15	55.6 (d)					
16	179.2 (s)	15				

[a] D_2O + CD_3OD, 75.5 MHz. [b] Assignments are based on extensive 2D NMR measurements (HMBC, HSQC, COSY). [c] Implied multiplicities determined by DEPT (C = s; CH = d; CH_2 = t; CH_3 = q). [d] Numbers refer to proton resonances.

The molecular formula $C_{11}H_{15}N_3O_3$ was deduced for compound **3** by accurate mass measurement. Comparison of its ^{1}H and ^{13}C NMR spectral data with those for compound **2** (see Tables 1 and 2) clearly showed it to have a urocanic acid moiety. ^{1}H−^{1}H COSY correlations observed between H_2-7 and H_2-8, between H_2-8 and H_2-9, and between H_2-9 and H_2-10, in conjunction with a long-range CH coupling observed between C-11 and H_2-10, and a downfield chemical shift for the resonance of H_2-7 (δ 3.16) clearly indicated the second part of the molecule to be a 5-aminopentanoic acid moiety. An HMBC coupling between C-6 and H_2-7 proved the linkage between the 5-aminopentanoic acid moiety and the urocanic acid moiety via an amidic bond.

High-resolution electron-impact mass spectometry (HREIMS) analysis of compound **4** gave a molecular formula of $C_{27}H_{27}N_7O_7S$, with 18 degrees of unsatura-

Table 3. ^{1}H NMR and ^{13}C NMR Spectral Data for Compounds **4** and **5**[a]

	4			**5**		
carbon	δ_C (mult)[b]	δ_H (mult, J in Hz)[b]	HMBC[d]	δ_C (mult)[b]	δ_H (mult, J in Hz)[b]	HMBC[d]
1	157.2 (s)[c]		2	157.5 (s)		2
2	154.1 (d)	8.29 (1H, s)		154.2 (d)	8.31 (1H, s)	
3	150.5 (s)		2, 5, 6	150.4 (s)		2, 6
4	120.3 (s)		2, 5	120.8 (s)		5
5	141.4 (d)	8.43 (1H, s)	6	142.1 (d)	8.37 (1H, s)	6
6	87.7 (d)	6.40 (1H, d, J = 5.7)	7, 8	89.1 (d)	6.45 (1H, d, J = 5.5)	7, 8
7	74.9 (d)	6.23 (1H, t, J = 5.7)	6	74.7 (d)	6.31 (1H, t, J = 5.5)	6
8	73.9 (d)	5.87 (1H, dd, J = 4.5, 5.7)	9	74.4 (d)	6.02 (1H, t, J = 5.5)	9
9	83.4 (d)	4.62 (1H, m)	10	77.8 (d)	4.87 (1H, m)	10
10	37.0 (t)	3.10 (1H, d, J = 5.5) 3.12 (1H, d, J = 5.5)	9, 11	55.5 (t)	3.58 (2H, d, J = 5.8)	11
11	16.6 (q)	2.21 (3H, s)		39.3 (q)	2.76 (3H, s)	
12	164.7 (s)		7, 14	164.6 (s)		7, 14
13	142.5 (s)		21	143. 0 (s)		21
14	128.2 (d)	6.97 (1H, s)	16, 20	127.8 (d)	7.00 (1H, s)	16, 20
15	125.1 (s)		17, 19	125.5 (s)		17, 19
16	133.5 (d)	7.54 (1H, d, J = 8.7)	20	133.5 (d)	7.54 (1H, d, J = 8.5)	20
17	116.7 (d)	6.70 (1H, d, J = 8.7)	19	116.6 (d)	6.72 (1H, d, J = 8.5)	19
18	160.5 (s)		16, 17, 19, 20	160.6 (s)		16, 17, 19, 20
19	116.7 (d)	6.70 (1H, d, J = 8.7)	17	116.6 (d)	6.72 (1H, d, J = 8.5)	17
20	133.5 (d)	7.54 (1H, d, J = 8.7)	16	133.5 (d)	7.56 (1H, d, J = 8.5)	16
21	59.6 (q)	3.65 (3H, s)	14	59.5 (q)	3.67 (3H, s)	
22	167.9 (s)		8, 23, 24	167.4 (s)		8, 24
23	114.3 (d)	6.58 (1H, d, J = 15.7)	24	114.5 (d)	6.58 (1H, d, J = 15.7)	24
24	138.8 (d)	7.76 (1H, d, J = 15.7)		139.0 (d)	7.78 (1H, d, J = 15.7)	
25	133.2 (s)			133.1 (s)		
26	125.0 (d)	7.42 (1H, s)		123.1 (d)	7.41 (1H, s)	
27	139.2 (d)	7.84 (1H, s)	26	139.2 (d)	7.84 (1H, s)	26

[a] CD_3OD, 300 MHz (^{1}H); 75.5 MHz (^{13}C). [b] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [c] Implied multiplicities determined by DEPT (C = s; CH = d; CH_2 = t; CH_3 = q). [d] Numbers refer to proton resonances.

tion. From the ^{1}H NMR and ^{13}C NMR spectra it was evident that a nucleoside moiety was present in the molecule. Characteristic chemical shifts of C-1, C-2, C-3, C-4, and C-5 (see Table 3) clearly established the purine to be adenine. ^{1}H−^{1}H COSY couplings observed between H-6 and H-7, between H-7 and H-8, between H-8 and H-9, and between H-9 and H_2-10 showed the sugar moiety to be a pentose. The linkage between the sugar moiety and the adenine moiety was evident from an HMBC coupling observed between C-5 and H-6. A long-range CH coupling between C-10 and H_3-11 and the unusual ^{1}H and ^{13}C NMR chemical shifts for CH_3-11 and CH_2-10 (see Table 3) were indicative of a methylthioadenosine moiety. Downfield chemical shifts for the resonances of H-7 (δ 6.23) and H-8 (δ 5.87) showed both of oxygen atoms attached to C-7 and C-8 to be α to carbonyl carbons, a fact that was supported by HMBC couplings observed between carbonyl C-22 and H-8 and between carbonyl C-12 and H-7. Additional long-range CH couplings between C-22 and H-23, between C-22 and H-24, and between C-27 and H-26, ^{1}H−^{1}H coupling between H-23 and H-24 (J = 15.7 Hz; trans double bond), as well as a comparison of ^{1}H and ^{13}C NMR chemical shifts for CH-23, CH-24, C-25, CH-26, and CH-27 with those of compounds **2** and **3** gave evidence of the linkage of a urocanic acid moiety to C-8. C-12 has to be connected to double bond Δ^{13} because of a long-range CH coupling observed between C-12 and H-14. An HMBC coupling between C-13 and H_3-21 allowed the methoxyl group to be linked to C-13. At this point a comparison of the ^{1}H and ^{13}C NMR chemical shifts of C-15, CH-16, CH-17, C-18, CH-19, and CH-20 with those of compound **1** clearly established the presence of a para-hydroxylated benzene ring. The benzene ring has to be connected to C-14 because of HMBC couplings

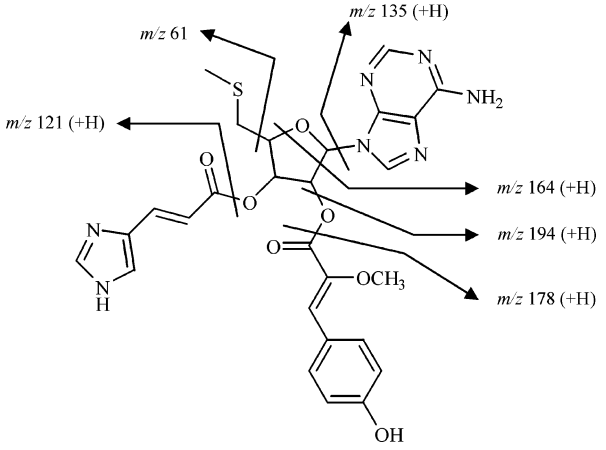


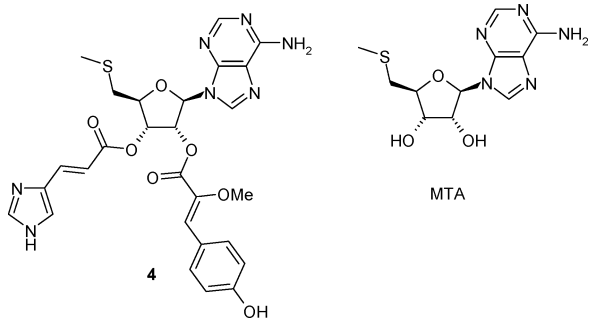
Figure 1. EI-MS fragmentation of compound **4**.

between C-14 and both H-16 and H-20 and thus completed the 3-(4-hydroxyphenyl)-2-methoxy-2-propenoic acid moiety attached to C-7. The planar structure of **4** was in good agreement with the EI-MS fragmentation pattern (see Figure 1).

The geometry of the double bond Δ^{13} was assigned on the basis of the chemical shifts for C-14 (δ 128.2) to be *Z* as in compound **1**. The relative stereochemistry of the pentose was deduced from 2D ^{1}H−^{1}H nuclear Overhauser effect spectrometry (NOESY) correlations and ^{1}H−^{1}H NMR coupling constants. A NOESY cross-peak between H-6 and H-9 showed both protons to be on the same side of the molecule. This fact and the characteristic ^{1}H−^{1}H coupling constants of H-6, H-7, and H-8 (see Table 3) were indicative of ribose.[7]

Compound **5** was obtained as a white solid. Its molecular formula was deduced from accurate FABMS to be $C_{27}H_{27}N_7O_8S$. Comparison of the ^{13}C NMR data

Table 4. Affinities of Compound **4** at Adenosine A_1, A_{2A}, A_{2B}, and A_3 Receptors



	$K_i \pm$ SEM (μM)				
compd	A_1[a] vs [^{3}H]CCPA	A_1[b] vs [^{3}H]DPCPX	A_{2A}[c] vs [^{3}H]MSX-2	A_{2B}[d] vs [^{3}H]PSB-298	A_3[e] vs [^{3}H]PSB-11
4	3.26 ± 0.45	9.45 ± 0.08	17.1 ± 1.74	>10[f]	6.94 ± 0.16
MTA	0.15 ± 0.01 (0.24)[69,g]	0.21 ± 0.001	1.13 ± 0.31 (1.18)[69,h]	13.9 ± 3.21[j] (8.2)[18,i]	0.68 ± 0.03

[a] Displacement of [^{3}H]CCPA from rat cortical membranes. [b] Displacement of [^{3}H]DPCPX from rat cortical membranes. [c] Displacement of [^{3}H]MSX-2 from rat striatal membranes. [d] Displacement of [^{3}H]PSB-298 from the human A_{2B} receptor expressed in HEK-293 cells. [e] Displacement of [^{3}H]PSB-11 from the human A_3 receptor expressed in CHO cells. [f] Less than 10% inhibition of radioligand binding at a concentration of 10 μM. [g] Binding vs [^{3}H]R-PIA instead of [^{3}H]CCPA as radioligand. [h] Binding vs [^{3}H]NECA instead of [^{3}H]MSX-2 as radioligand. [i] Human A_{2B} receptors in VA13 fibroblasts, inhibition of adenylate cyclase. [j] Value obtained by extrapolating the curve because of limited supply of the compound.

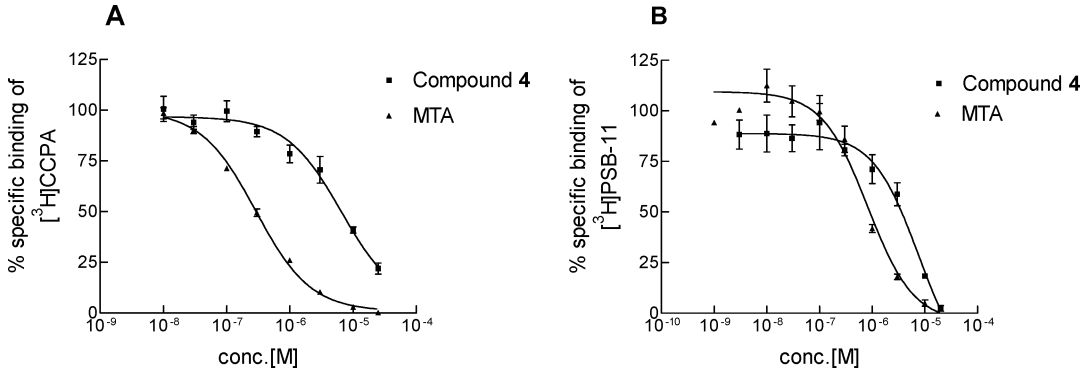


Figure 2. (A) Adenosine receptor binding curve of compound **4** and MTA at A_1ARs. Displacement of [^{3}H]CCPA was from rat brain cortical membranes. (B) Adenosine receptor binding curve of compound **4** and MTA at A_3ARs. Displacement of [^{3}H]PSB-11 from human A_3 adenosine receptors was expressed in Chinese hamster ovary (CHO) cells.

with those of compound **4** showed the structure of **5** to be very similar to that of **4**. The only striking differences in the ^{13}C NMR spectrum are the chemical shifts for C-10 and C-11, indicating the presence of a sulfoxid group in **5**. Concerning coupling constants of H-6, H-7, and H-8, and comparable optical rotation values for compound **4** (−115.0) and compound **5** (−124.1), we deduce the sugar to be ribose.

N,*N*-Dimethylglycine (**6**), to our knowledge, is described here for the first time as a natural product.[27] 4-Hydroxybenzoylcholin (**7**) in the terrestrial environment has been isolated from *Sinapsis alba*[28] but not from the marine habitat. α-D-Mannosylpyranosyl-L-tryptophan (**8**) was first isolated in 2000 from the marine ascidians *Leptoclinides dubius* and *Pharyngodictyon cauliflos*.[29] The absolute stereochemistry of **8** was determined by circular dichroism. 2-Piperidinon (**9**), which in the marine habitat was isolated from the sponge *Tedania ignis*,[30] is a cyclic 5-aminopentanoic acid, which is part of compound **3.** From polar vacuum liquid chromatography (VLC) fractions, amino acids such as valine and isoleucine, taurine, and the *N*-methylpyridinium alkaloids homarine (**11**) and sulcatin (**10**) were isolated. Taurine and homarine are widespread marine natural products.[31,32] Sulcatin, to date, was only reported from the ascidian *Micrococosmus vulgaris*.[3] Further separation of polar VLC fractions yielded numerous widespread purines and nucleosides: thymidine, inosine, deoxyinosine, uridine, deoxyuridine, adenine, and xanthine.

Biological Evaluation. Adenosine derivative **4** was investigated in radioligand binding assays in order to determine its affinity for adenosine A_1, A_{2A}, A_{2B}, and A_3 receptor subtypes (Table 4, Figure 2). For A_1 receptors the agonist radioligand [^{3}H]CCPA and the antagonist radioligand [^{3}H]DPCPX were used. For the A_{2A} receptors the selective antagonist [^{3}H]MSX-2 was taken as a radioligand. A_{2B} receptor affinity was determined using the antagonist radioligand [^{3}H]PSB-298. Affinities for the A_3 receptors were established by applying the antagonist radioligand [^{3}H]PSB-11. In Table 4 the affinities of the investigated compound **4** at the different adenosine receptor subtypes are shown. For comparison, data of the related compound, 5′-deoxy-5′-methylthioadenosine (MTA), are included.

Compound **4** exhibited binding affinities in the micromolar range at A_1, A_{2A}, and A_3 adenosine receptors (K_i values of 3.26, 17.1, and 6.94 μM), whereas no significant binding affinity could be observed at A_{2B} adenosine receptors at the concentrations tested (0.001−

10 μM) ($K_i \gg 10$ μM). Compound **4** exhibited approximately 3-fold higher affinity at the A_1AR versus the agonist [^{3}H]CCPA than versus the antagonist radioligand [^{3}H]DPCPX. It has been reported previously that agonistic ligands show lower affinity versus antagonist radioligands,[34] indicating that **4** may be an agonist at A_1ARs. The related 5′-deoxy-5′-methylthioadenosine exhibited binding affinities in the high nanomolar range at A_1ARs and A_3ARs (K_i values of 0.15 and 0.68 μM, respectively) and in the micromolar range at A_{2A}ARs and A_{2B}ARs (K_i values of 1.13 and 13.9 μM, respectively). Previously published data for MTA at ARs are well in accordance with our data (see Table 4). Compound **4** consists of a nucleoside moiety (adenosine derivative) with a methylthio group at the 5′-position of the ribose moiety instead of a hydroxyl group. The ribose is esterified with 3-(4-hydroxyphenyl)-2-methoxyacrylic acid at the 2′-position and with urocanic acid at the 3′-position. It is generally believed that the ribose moiety is essential (i) for high affinity of adenosine derivatives and (ii) for their agonistic activity at ARs; 2′,3′-dideoxyadenosine derivatives are weak AR antagonists.[35−37] 5′-Deoxy-5′-methylthioadenosine has previously been described as an agonist[15] or a partial agonist[16] at A_1ARs, a partial agonist at A_{2A}ARs,[17] and an antagonist[15,18] or a weak partial agonist[17] at A_{2B}ARs. Data for MTA at A_3ARs have not been available. The comparison of the binding affinities obtained for compound **4** and MTA showed that MTA with its unsubstituted 2′- and 3′-hydroxyl group exhibited higher binding affinities than compound **4** at A_1, A_{2A}, A_{2B}, and A_3ARs. Therefore, it can be confirmed that the existence of the 2′- and 3′-hydroxyl groups is indeed important for high adenosine receptor affinity. Only few adenosine derivatives with substitutions at the 2′- and/or 3′-hydroxyl groups have so far been investigated at ARs.[38−41] The 2′-methoxy-substituted N^6-cyclohexyladenosine (SDZ-WAG-994) was reported to be a full and selective agonist at A_1ARs in vitro;[42] however, it was 10-fold less potent than the parent compound N^6-cyclohexyladenosine (CHA) but somewhat more A_1-selective.

2′,3′-Epoxide derivatives showed decreased binding affinity at A_3ARs, and agonists turned into antagonists after epoxidation.[43] The epoxidation of the two hydroxyl groups of the potent agonist 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (Cl-IB-MECA) led to a decrease in binding affinity by more than 1000-fold, and the efficacy was dramatically decreased to only ~7% of the maximal efficacy of the parent compound. This indicates the important role of one or both of the hydroxyl groups in binding as well as in the activation process at human A_3ARs.[43] The substitution of one of the hydroxyl groups of the ribose moiety with an amino group had yielded 3′-amino-3′-deoxyadenosine as the most potent A_3 agonist in a series of other amino-substituted adenosine derivatives.[44] 3′-Amido-substituted (5′-deoxy)-N^6-cyclopentyl-adenosines had been reported to be antagonists at rat A_1 adenosine receptors.[45]

To investigate the functional properties of the new nucleoside diester **4**, A_1 and A_3 adenosine receptor displacement experiments were performed both in the absence and in the presence of GTP in order to determine the so-called GTP shift, which is an in vitro parameter indicating intrinsic activity.[46] GTP can cause an uncoupling of the receptor from the G protein, leading to a shift of the receptor from the high- to the low-affinity state for agonists. The A_1AR affinity of compound **4** determined at rat brain cortical membranes in the absence and in the presence of GTP is shown in Table 5. The addition of GTP (100 μM) resulted in a rightward shift of the binding curve of **4**, and the IC_{50} value was increased. The GTP shift of 6.2 at A_1 adenosine receptors indicated that this compound behaves as a partial agonist at A_1 adenosine receptors. The GTP shift was even greater for MTA (22.2), indicating that MTA may exhibit even higher intrinsic activity than its diester derivative **4** (see Table 5).

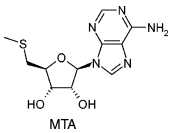
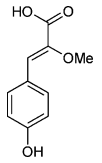
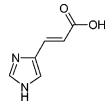
Table 5. Affinities (IC_{50} Values in the Presence and Absence of GTP) and GTP Shifts of Compound **4** in Comparison with Standard Compounds

	A_1 adenosine receptors[a]		
compd	IC_{50} − GTP (μM)	IC_{50} + GTP (μM)	GTP shift[b]
4	23.0 ± 0.2	143 ± 53	6.2 ± 0.1[c] (n = 2)
MTA	0.68 ± 0.11	15.1 ± 4.5	22.2 ± 0.02[c] (n = 6)
CPA (full agonist)	0.005 ± 0.002	0.125 ± 0.02	22.8 ± 0.02[c] (n = 3)
9-methyladenine[70] (antagonist)	12.5 ± 2.9[70]	14.7 ± 2.8[70]	1.2 ± 0.4[70]

[a] Displacement of [^{3}H]DPCPX from rat cortical membranes expressed as IC_{50} (μM). [b] The affinities for the adenosine A_1 receptor were determined in the absence and presence of 100 μM GTP. The GTP shifts were calculated by dividing the IC_{50} values determined in the presence of GTP by those measured in the absence of GTP. [c] Shift values were significantly different from 1.0 ($P \leq 0.05$).

To further investigate whether compound **4** acts as an agonist or an antagonist at A_3ARs, a functional assay was employed. A_3ARs are coupled to adenylate cyclase as a second messenger system in an inhibitory manner. The inhibition of forskolin-stimulated cAMP accumulation by compound **4** and MTA in comparison with the agonist NECA was determined in CHO cells expressing the human A_3AR (see Table 7). The A_3AR agonist NECA dose-dependently inhibited cAMP accumulation with an IC_{50} value of 66 μM. Compound **4** and MTA both showed the same effects with IC_{50} values of 6.7 and 71 μM, respectively. The IC_{50} value obtained for compound **4** in the functional assay was consistent with its K_i value from radioligand binding. However, the IC_{50} values for the standard compound NECA and for MTA were considerably higher than their affinities determined in binding studies. Such discrepancies have been described before and explained by an ineffective receptor−effector coupling in membrane preparations of CHO cells expressing A_3ARs.[47] The intrinsic activities, given relative to the intrinsic activity of NECA (set at 100%), are 56%

Table 6. CE Measurements of Hydrolysis Products of Compound **4** after Incubation with Esterase[a]

peak no.	mean migration time	structures of products	λmax in UV spectrum (nm)
A	17.72 ± 0.31	**4**[b]	295
B	4.77 ± 0.06	 MTA	260
C	5.80 ± 0.07		283
D	6.28 ± 0.08		275

[a] For incubation conditions, see Figure 3 and Experimental Section. [b] 85% of compound **4** was unhydrolyzed.

Table 7. Inhibition of Forskolin-Stimulated Adenylate Cyclase in Membrane Preparations of Chinese Hamster Ovary (CHO) Cells Recombinantly Expressing the Human A_3 Adenosine Receptor

compd	inhibition of forskolin-stimulated cAMP accumulation in CHO cell membranes expressing the human A_3AR IC_{50} ± SEM (μM)	efficacy (%) relative to NECA (=100%)
NECA	65.6 ± 14.2 (n = 6)	100
MTA	71.0 ± 42.0 (n = 3)	46 ± 9
4	6.7 ± 3.1 (n = 3)	56 ± 10

for compound **4** and 46% for MTA. Therefore, compound **4** appears to be somewhat more potent and efficacious in inhibiting adenylate cyclase than the related compound MTA. In conclusion, compound **4** clearly exhibits agonistic activity at human A_3ARs, and it can be described as a relatively potent partial agonist at A_3ARs in comparison to the standard agonist NECA. These results are in line with previous observations that 5′-deoxy-5′-alkylthio-substituted analogues of N^6-benzyl- or 3-iodobenzyladenosine are selective partial agonists for the human A_3 adenosine receptor.[48]

To exclude the possibility of ester hydrolysis of compound **4** to yield MTA and to make sure that the measured effects were actually due to intact compound **4**, we examined a possible hydrolysis of compound **4** by rat brain cortical membrane preparations as a potential source of esterase activity. Compound **4** was incubated with rat brain cortical membranes under the same conditions as in the radioligand binding assays. By means of micellar electrokinetic chromatography (MECC), the potential hydrolysis was examined. The resulting electropherogram exhibited only a single peak that could be identified as the nonhydrolyzed intact compound **4** by comparison with the electropherogram of a previously tested standard solution. The migration time for compound **4** was found to be 17.72 min with maximum absorption at 295 nm. Therefore, it could be confirmed that no hydrolysis of compound **4** occurred when incubating it with rat brain cortical membranes, and the obtained affinities in radioligand binding studies were from the intact esterified compound **4**, which is stable under the conditions of radioligand binding studies. Furthermore, the stability of compound **4** was examined by heating a 25 μM solution of the compound in Tris-HCl buffer 50 mM, pH 7.4, at 99 °C for 10 min. When this solution was injected into the CE instrument, no degradation products could be detected, demonstrating that the compound is quite stable at high temperature. To examine the enzymatic hydrolysis of compound **4**, it was incubated with carboxylesterase, which should be able to cleave the ester linkages at the 2′- and/or 3′-positions. As expected, compound **4** was hydrolyzed at both ester linkages, yielding three products with peaks appearing at 4.77, 5.80, 6.28, and 17.72 min, respectively, in the electropherogram (Figure 3). The latter peak appeared at the same migration time as the standard and could be identified as the intact compound **4**. However, it must be noted that the cleavage of compound **4** by the esterase was not quantitative and quite slow under the applied conditions (only approximately 10% of compound **4** was hydrolyzed after 1 h). Further confirmation of the structures of the hydrolyzed products was obtained by their UV spectra, each peak showing a different UV spectrum. Wavelengths of maximum absorption are collected in Table 6. In adenosine A_1 receptor binding assays of compound **4** performed in the presence of carboxylesterase, binding affinities in the nanomolar range (K_i = 330 ± 40 nM; n = 2) were determined, indicating a partial cleavage of the compound (compare K_i values in Table 4).

Molecular Modeling Studies. Molecular modeling studies were performed in order to explain and rationalize the obtained pharmacological results by theoretical receptor models and to improve and refine our models on the basis of the new data. The 3D structures of the A_1AR and the A_3AR were constructed using homology modeling with bovine rhodopsin (bRh) as a template. This is to date the only available X-ray structure of a G-protein-coupled receptor.[19] The root-mean-square distance between the backbone atoms in the α-helices of the template and the A_1AR model are 1.02 Å (inactive state) and 1.37 Å (active state), respectively. The corresponding root-mean-square distance values for the A_3AR model are 1.02 (inactive state) and 1.27 Å (active state).

The putative active conformations of the A_1AR and the A_3AR were built by modifying the conformation of tryptophan 247 (χ_1 from −65° to 174° and χ_2 from −79° to −96°). In this way the bulky side chain moves into the gap between transmembrane helix 5 (TM5) and TM6. The reason for this modification is that for the human A_3AR it was found that the corresponding mutant W243A (tryptophan → alanine) receptor was able to bind agonists but failed to activate the receptor.[49] Therefore, it can be concluded that the major difference in the shape of the binding pocket for agonists and antagonists is the absence of the bulky indole residue. The easiest way to achieve this, without major movement of the whole helix, is the above-mentioned movement of the side chain.

In a UV absorption study,[50] it was suggested that Trp265 in bRh (corresponding to Trp243 in the human A_3AR) tilts toward the membrane plane during conversion of the inactive to the active state (MI → MII) of the receptor. This is consistent with the proposed conformational change for Trp in TM6 of adenosine

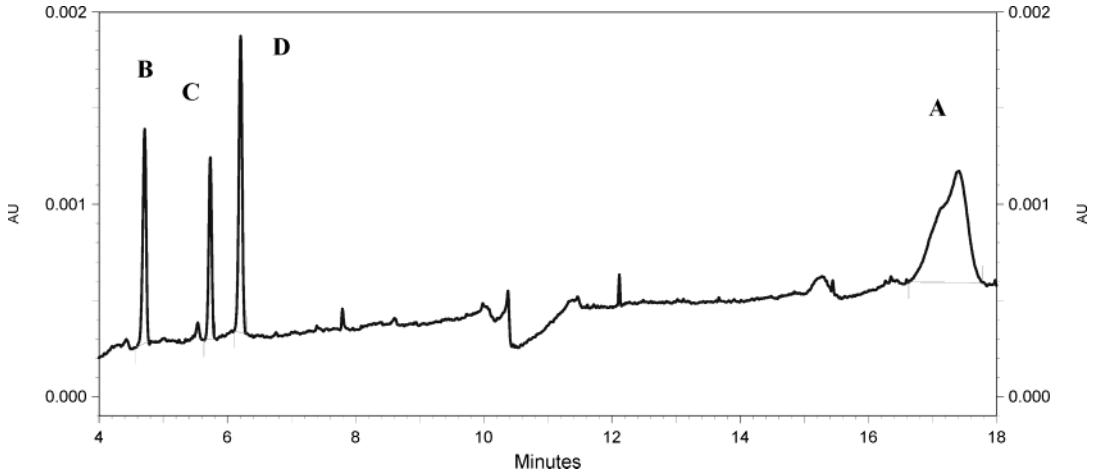


Figure 3. Electropherogram of compound **4** (concentration 25 μM) incubated with carboxylesterase from porcine liver (2 μL) at pH 8.0 and 25 °C for 60 min. The separation conditions were 25 mM sodium borate buffer, 100 mM SDS, pH 8.5, fused silica capillary, 40 cm length (30 cm to the detector), 75 μM i.d., 95 μA, 25 °C, detection at 260 nm, 0.1 psi pressure injection (25 s).

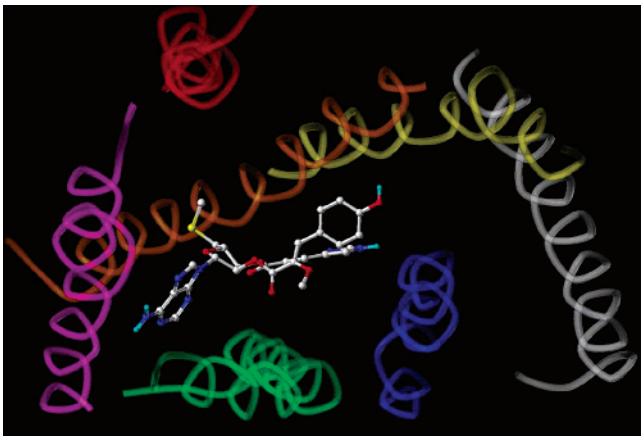


Figure 4. Extracellular view of the structure of compound **4** docked into the A_3AR. The transmembrane helices are shown as transparent tubes: TM1, white; TM2, yellow; TM3, orange; TM4, red; TM5, magenta; TM6, green; TM7, blue.

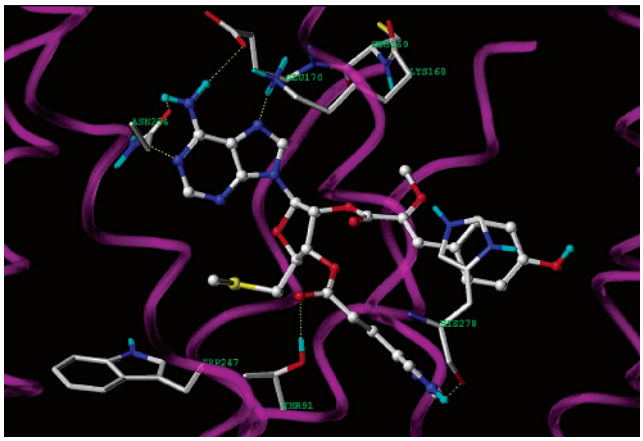


Figure 5. Compound **4** docked into the binding site of the active conformation of the human A_1 adenosine receptor. Coloration of the ligand is according to the atom type. Possible H-bonds are shown as yellow dashed lines. The transmembrane helices are displayed as transparent magenta tubes.

receptors. Very recently Nikiforovich and Marshall proposed a three-dimensional model for meta-II rhodopsin featuring a similar change to the conformation of Trp265.[51] A docking study by Gao et al. for agonists at the A_3AR led to similar values for χ_1 and χ_2.[43] This tryptophan is highly conserved within the large group of the rhodopsin-like receptors (class A GPCRs). The most frequent modification is Tyr or Phe (e.g., G-protein-coupled P2Y receptors). These aromatic amino acids may be able to fulfill the same role as tryptophan. There is a small number of class A receptors that are more different, including hormone protein receptors (Trp → Met), olfactory receptors (Trp → Ala), prostacycline receptors (Trp → Ser), and GPR/endothelin B-like receptors (Trp → Ile).[52] It appears likely that tryptophan in this position plays a crucial role in receptor activation for many but not all GPCRs.

Compound **4** acted as a partial agonist at A_1 and A_3ARs. Figure 4 shows compound **4** docked into the active conformation of the A_3AR viewed from the extracellular side. TM3, TM5, TM6, and TM7 appear to be involved in ligand binding. This is consistent with several mutagenesis studies carried out on different AR subtypes that indicated direct ligand interactions with TMs 3, 6, 7 and the second extracellular loop.[49,53,54]

Figure 5 shows compound **4** docked into the active conformation of the A_1AR model. There are possible H-bonds to Asn254 (TM6), Thr91 (TM3), the backbone oxygen of His278 (TM7), Lys168 (EL2), and Glu170 (EL2). By comparision of these docking results with those obtained for the docking of **4** into the active conformation of the A_3AR (Figure 6), it can be seen that the ligand binds in roughly the same orientation. The small differences consist mainly of the absolute position of the ligand in the binding pocket. A possible reason for these slight differences in the binding modes could be the exchange of the probably H-bonded Gln92/Asn184 (in the A_1AR) to His95/Ser181 in the A_3AR. This pair occupies a slightly larger space in a position where the docking of **4** to the A_1AR already showed an overlap of van der Waals radii.

Further docking experiments using the inactive receptor conformations revealed that compound **4** is able to fit into both active and inactive receptor conformations of the A_1AR (Figure 7) as well as the A_3AR (Figure 8). The fitness scores calculated by the GOLD software for the docking of compound **4** into the respective active and inactive states of the receptors are similar, a result that is consistent with the experimentally observed partial agonism of the compound. However, the orienta-

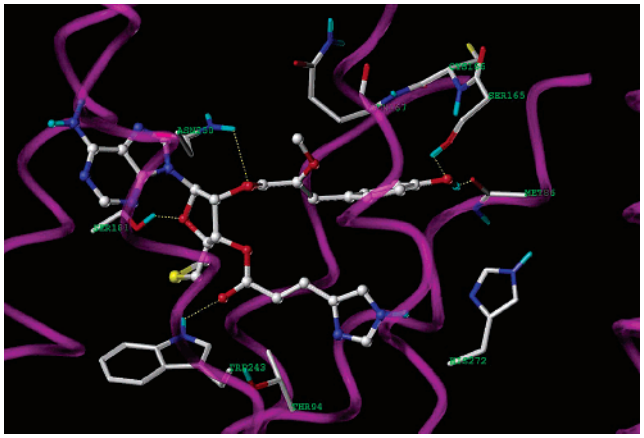


Figure 6. Compound **4** docked into the binding site of the active conformation of the human A_3 adenosine receptor.

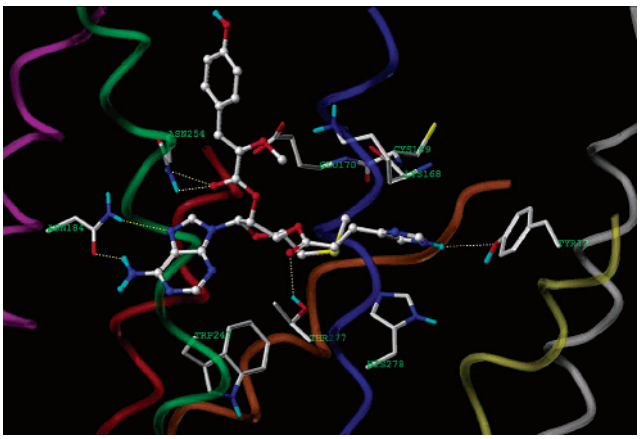


Figure 7. Compound **4** docked into the binding site of the inactive conformation of the human A_1 adenosine receptor.

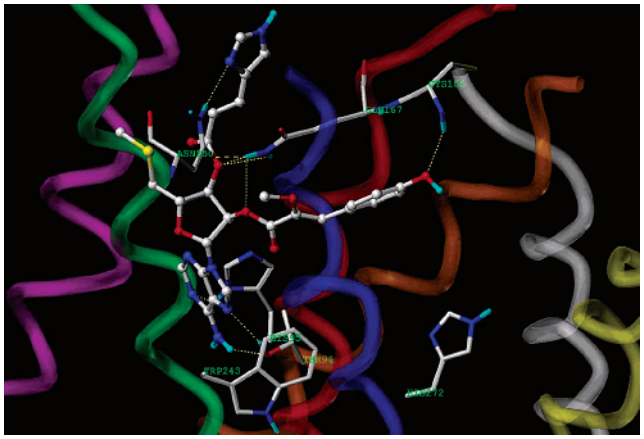


Figure 8. Compound **4** docked into the binding site of the inactive conformation of the human A_3 adenosine receptor.

tions of the ligand in the binding site of the inactive ARs (Figures 7 and 8) are completely different from that found for the active A_1 and A_3AR (Figures 5 and 6). Furthermore, the binding modes of compound **4** in the inactive A_1AR and A_3AR are less similar than for the active A_1 and A_3AR states. This observation suggests that the active conformation of both adenosine receptor subtypes tends to recognize the ligand in the same way, whereas the inactive receptor conformation is less restrictive and allows different binding modes.

Although compound **4** is quite bulky, it was possible to dock it into both receptor models (A_1 and A_3). It is often much easier to dock smaller molecules into a binding pocket. Therefore, this docking study can be taken as one step in the validation of these receptor models. The binding characteristics of compound **4**, which can be related to its partial agonistic properties, could lead to a further refinement of the receptor models.

Conclusion

Five new amino acid derived structures (**1**−**5**) were isolated from the methanolic extract of the Australian ascidian *Atriolum robustum*. Elucidation of the structures by employing spectroscopic techniques revealed that structures **4** and **5** are complex nucleosides containing rare methylthioadenosine and methylsulfinyladenosine moieties.

Compound **4** exhibited affinity for A_1 and A_3 adenosine receptors in the low micromolar concentration range. Its affinity was lower at A_{2A} and much lower at A_{2B} ARs. GTP shift experiments (A_1) and the inhibition of cAMP accumulation (A_3) indicated that compound **4** behaves as a partial agonist at A_1 and A_3 adenosine receptors. Because of its agonism at A_1 adenosine receptors, compound **4** may exhibit analgesic, sedative, and cardiac depressant activities. Partial A_1 agonists appear to be advantageous for therapeutic use because of limited side effects in comparison with full agonists.[55] The fact that two of the secondary hydroxyl groups are esterified might be an advantage for its use as a biologically active agent. The increased lipophilicity could result in a better absorption, might be responsible for a longer half-life, and may also enhance penetration of the blood−brain barrier, which is required for central nervous system (CNS) effects. The results obtained from ester hydrolysis experiments showed that compound **4** in addition to its own pharmacological activity might act as a lipophilic prodrug of 5′-deoxy-5′-methylthioadenosine in vivo, which can be slowly released by esterases and which is about 10-fold more potent than **4**.

The sterically demanding compound **4** was docked into homology models of the A_1 and A_3 adenosine receptor. Because of its highly flexible structure, compound **4** fits well into the binding pockets of both receptor subtypes.

Compound **4**, which was found to be inactive against several tumor cell lines at the 10 μg/mL level, may serve as a new nature-derived lead compound for the development of partial A_1 and A_3 agonists and/or prodrugs thereof.

Experimental Section

General Experimental Procedures. HPLC was carried out using a Merck-Hitachi system consisting of a L-6200 A pump, a L-4500 A photodiode array, and a Knauer K-2300 differential refractometer as detectors together with a D-6000 A interface. ^{1}H, ^{13}C, COSY, NOESY, HSQC, and HMBC NMR spectra were recorded using a Bruker Avance 300 DPX spectrometer in D_2O and MeOD. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 3.35/49.0 (MeOD). UV and IR spectra were obtained by employing Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. GC−MS analyses were performed with a Perkin-Elmer AutoSystem XL and a TurboMass spectrometer. Optical rotations and CD spectra were measured on Jasco DIP 140 and J-715 polarimeters, respectively. HRE-IMS data were recorded on a Finnigan MAT 95 spectrometer

and HRFABMS (mNBA) data on a JEOL 102SX A spectrometer. All other experimental details were as previously reported.[56]

Animal Material. *Atriolum robustum* was collected in July of 1988 by scuba diving at Heron Islands, Wistari Reef, the Great Barrier Reef, Australia, from a depth of 20 m and stored at −20 °C until required. A voucher specimen is deposited at the Institute for Pharmaceutical Biology, University of Bonn (voucher number HER31).

Extraction and Isolation. After removal of the preservation EtOH, the ascidian (wet weight of 120 g) was extracted with MeOH (3 × 0.5 L). The MeOH extract and the EtOH solution were combined and evaporated to dryness to yield 5.6 g of a salty, brownish extract. This extract was fractionated by vacuum liquid chromatography (VLC) over Polygoprep 60−50 C_{18} material (Macherey-Nagel) using gradient elution from H_2O (100%) to MeOH (100%) to give 12 fractions. All these fractions were desalinated using solid-phase extraction (Bakerbond SPE C_{18}), and then fractionated by reversed-phase (RP) HPLC.

Fraction 1 was rechromatographed by RP-HPLC (Knauer NH_2 Eurospher-100 column, 250 mm × 8 mm, 5 μm, MeOH/H_2O (0.5:9.5, 0.05% acetic acid), 1.5 mL/min). Purification with a Knauer diol Eurospher-100 column (250 mm × 8 mm, 5 μm, MeOH/CH_2Cl_2 (1:1), 1.5 mL/min) gave 15.3 mg of taurine as a white powder, 22.3 mg of **11** as a colorless solid, and 11.2 mg of **6** as a white solid. HPLC separation and purification of fraction 2 (separation by Knauer NH_2 Eurospher-100, 250 mm × 8 mm, 5 μm, MeOH/H_2O (0.1:9.9, 0.05% acetic acid), 1.5 mL/min; purification by Knauer diol Eurospher-100, 250 mm × 8 mm, 5 μm, MeOH/CH_2Cl_2 (3:7), 1.5 mL/min) yielded compound **10** (1.9 mg) as a colorless solid.

Fraction 3 was rechromatographed with a Knauer NH_2 Eurospher-100 column (250 mm × 8 mm, 5 μm; MeOH/H_2O (0.1:9.9; 0.05% acetic acid), 1.5 mL/min). After purification with a Knauer diol Eurospher-100 column (250 mm × 8 mm, 5 μm; MeOH/CH_2Cl_2 (3:7), 1.5 mL/min) compounds **3** (34.3 mg, white solid) and **8** (4.8 mg, white solid, $[\alpha]^{23}_D$ +6.5° (*c* 0.34, H_2O)),[12] adenine (1.3 mg, white solid), uridine (2.0 mg, white solid), and deoxiuridine (0.9 mg, white solid) were obtained. Compound **2** (23.9 mg, white solid), compound **9** (3.2 mg, colorless solid), xanthine (4.3 mg, white solid), and thymidine (8.7 mg, white solid) were isolated from fraction 4 (separation by Knauer NH_2 Eurospher-100, 250 mm × 8 mm, 5 μm, MeOH/H_2O (2:8), 1.5 mL/min; purification by Knauer diol Eurospher-100, 250 mm × 8 mm, 5 μm, MeOH/CH_2Cl_2 (1:1), 1.5 mL/min). Separation of fraction 5 by RP-HPLC (Knauer NH_2 Eurospher-100, 250 mm × 8 mm, 5 μm, MeOH/H_2O (2:8), 1.5 mL/min) followed by purification (Knauer diol Eurospher-100, 250 mm × 8 mm, 5 μm, MeOH/CH_2Cl_2 (1:1), 1.5 mL/min) yielded compound **1** (1.8 mg, white solid) and inosine and deoxyinosine as a 1:1 mixture (1.2 mg, white solid).

Fraction 6 was subjected to RP-HPLC (Knauer RP-18 Eurospher-100, 250 mm × 8 mm, 5 μm, MeOH/H_2O (4:6), 1.5 mL/min) to obtain compound **7** (8.4 mg, colorless solid). Compound **5** (1.0 mg, white solid) was obtained from fraction 7 (Knauer RP-18 Eurospher-100, 250 mm × 8 mm, 5 μm, MeOH/H_2O (6:4), 1.5 mL/min). Purification of fraction 9 by RP-HPLC (Knauer RP-18 Eurospher-100, 250 mm × 8 mm, 5 μm, MeOH/H_2O (1:1), 1.5 mL/min) yielded compound **4** (2.2 mg, white solid).

Amino Acid Analysis by Chiral GC. An amount of 0.5 mg each of **1** and **2** was dissolved in 1 mL of 6 N HCl and hydrolyzed in a sealed vial at 104 °C for 20 h. After removal of HCl by repeated evaporation in vacuo, the hydrolysate was heated with acetyl chloride (150 μL) and ^{i}PrOH (500 μL) at 100 °C for 45 min. The mixtures were evaporated to dryness using a stream of N_2, and the residues were treated with pentafluoropropionic anhydride (0.3 mL) in CH_2Cl_2 (0.6 mL) at 100 °C for 15 min. Excess reagents were removed by evaporation under a stream of N_2, and the residues were resolved in CH_2Cl_2 (1 mL). The derivatized amino acids were then analyzed by GC−MS using an Alltech capillary Chirasil-Val column (25 m × 0.25 mm, 0.16 μm; program rate, column temperature held at 50 °C for 3 min, 50−180 °C at 4 °C/min; flow, 0.6 mL/min helium; injection temperature, 250 °C). The retention times of the *N*-pentafluoropropionyl isopropyl ester derivatives of the amino acids established the presence of L-arginine (53.1 min) for compound **1** and L-lysine (37.6 min) for compound **2**.

(2*S*)-2-Amino-5-{[(*Z*)-3-(4-hydroxyphenyl)-2-methoxy-2-propenoyl]amino}(imino)methyl]amino}pentanoic acid (1): white solid (1.8 mg, 0.002%); $[\alpha]^{25}_D$ −3.2° (*c* 0.15, H_2O); UV (H_2O) λ_{max} (ϵ) 295 nm (34 000); IR (ATR) ν_{max} 3340, 3175, 2937, 1652, 1581, 1510, 1447, 1394, 1240, 1171, 1022 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); FABMS *m*/*z* 373 $[M + Na]^+$, 351 $[M + H]^+$; HRFABMS *m*/*z* 373.1488 $[M + Na]^+$ (calcd for $C_{16}H_{22}N_4O_5Na$, 373.1476).

(2*S*)-2-Amino-6-{[(*E*)-3-(1*H*-imidazol-4-yl)-2-propenoyl]-amino}hexanoic acid (2): white solid (23.9 mg, 0.02%); $[\alpha]^{25}_D$ −9.0° (*c* 1.7, H_2O); UV (H_2O) λ_{max} (ϵ) 280 nm (25 500); IR (ATR) ν_{max} 3276, 3072, 1660, 1614, 1573, 1409, 1350, 1018 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); FABMS *m*/*z* 289 $[M + Na]^+$, 267 $[M + H]^+$; HRFABMS *m*/*z* 265.1300 $[M - H]^-$ (calcd for $C_{12}H_{17}N_4O_3$, 265.1278).

5-{[(*E*)-3-(1*H*-Imidazol-4-yl)-2-propenoyl]amino}pentanoic acid (3): white solid (34.3 mg, 0.03%); UV (H_2O) λ_{max} (ϵ) 277 nm (39 500); IR (ATR) ν_{max} 3290, 3213, 2940, 1652, 1607, 1537, 1314, 1111, 1020, 962 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); FABMS *m*/*z* 260 $[M + Na]^+$, 238 $[M + H]^+$; HRFABMS *m*/*z* 238.1192 $[M + H]^+$ (calcd for $C_{11}H_{16}N_3O_3$, 238.1199).

(2*R*,3*R*,4*R*,5*S*)-2-(6-Amino-9*H*-purin-9-yl)-4-{[(*E*)-3-(1*H*-imidazol-4-yl)-2-propenoyl]oxy}-5-[(methylsulfanyl)methyl]tetrahydro-3-furanyl(*Z*)-3-(4-hydroxyphenyl)-2-methoxy-2-propenoate (4): white solid (2.2 mg, 0.002%); $[\alpha]^{24}_D$ −115.0° (*c* 0.18, MeOH); UV (MeOH) λ_{max} (ϵ) 293 nm (44 800); IR (ATR) ν_{max} 3333, 3124, 2923, 1707, 1634, 1601, 1510, 1427, 1248, 1090, 987 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 3); EIMS *m*/*z* (rel intens) 593 (0.2), 473 (3), 441 (2), 297 (6), 280 (5), 250 (7), 194 (40), 178 (5), 164 (100), 136 (67), 120 (25), 61 (7); HREIMS *m*/*z* 593.1693 $[M\cdot]^+$ (calcd for $C_{27}H_{27}N_7O_7S$, 593.1686).

(2*R*,3*R*,4*R*,5*S*)-2-(6-Amino-9*H*-purin-9-yl)-4-{[(*E*)-3-(1*H*-imidazol-4-yl)-2-propenoyl]oxy}-5-[(methylsulfinyl)methyl]tetrahydro-3-furanyl(*Z*)-3-(4-hydroxyphenyl)-2-methoxy-2-propenoate (5): white solid (1.0 mg, 0.001%); $[\alpha]^{24}_D$ −124.1° (*c* 0.08, MeOH); UV (MeOH) λ_{max} (ϵ) 288 nm (44 000); IR (ATR) ν_{max} 3125, 2934, 1694, 1635, 1601, 1511, 1435, 1329, 1303, 1271, 1217, 1170, 1088, 975 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 3); FABMS *m*/*z* 632 $[M + Na]^+$, 610 $[M + H]^+$; HRFABMS *m*/*z* 610.1720 $[M + H]^+$ (calcd for $C_{27}H_{28}N_7O_8S$, 610.1752).

Biological Assays. Cytotoxicity tests against the cell lines HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma), and MCF7 (breast adenocarcinoma) followed the standards of the NCI.[57]

Adenosine Receptor Binding Studies. The adenosine A_1 receptor binding assays were carried out at rat brain cortical membranes using the A_1-selective radioligand [^{3}H]CCPA (1.110 TBq/mmol, Amersham, Freiburg, Germany). The adenosine A_{2A} receptor binding assays were carried out at rat brain striatal membranes using the A_{2A}-selective radioligand [^{3}H]-MSX-2 (3.145 TBq/mmol, custom-labeled by Amersham, Germany). Both assays were performed as previously described.[58,59] Frozen rat brain membranes were obtained by Pel Freez, Rogers, AR, and thawed at 4 °C. Cortex and striatum were dissected, and membrane fractions were prepared. Protein concentrations of ca. 70 μg/mL were used in the assays, determined by the method of Lowry[60] using a Sigma Aldrich protein assay kit. Compound **4** was dissolved in DMSO. Inhibition curves were determined using seven to nine different concentrations of the test compound, spanning 3 orders of magnitude. In A_1 and A_{2A} receptor binding studies, three separate experiments were performed each in duplicate, unless otherwise noted.

The [^{3}H]CCPA concentration was 0.5 nM, and nonspecific binding was determined with 10 μM 2-chloroadenosine. Incu-

bations were carried out in a total volume of 1 mL of Tris-HCl buffer, 50 mM, pH 7.4, at room temperature (23 °C) for 90 min. Incubation was terminated by rapid filtration using a Brandel 48-channel cell harvester through Whatman GF/B glass fiber filters. Filters were rinsed three times with 2 mL each of ice-cold Tris-HCl buffer, 50 mM, pH 7.4.

[^{3}H]MSX-2 was used in a final concentration of 1 nM. Nonspecific binding was determined with 50 μM NECA. Incubations were carried out in a total volume of 1 mL of Tris-HCl buffer, 50 mM, pH 7.4, at room temperature (23 °C) for 30 min. Incubation was terminated by rapid filtration through GF/B glass fiber filters, presoaked in 0.5% aqueous polyethylenimine solution for 45 min to reduce nonspecific binding, using a Brandel 48-channel cell harvester. Filters were rinsed three times with 2 mL each of ice-cold Tris-HCl buffer, 50 mM, pH 7.4.

The adenosine A_{2B} receptor binding assay was carried out at HEK-293 cell membranes stably transfected with human A_{2B}-AR obtained from Receptor Biology Inc. via Perkin-Elmer Life Sciences, Germany.

The adenosine A_{2B} receptor binding assays were performed with [^{3}H]PSB-298 (4.59 TBq/mmol, Amersham, Germany) in a final concentration of 1 nM. Nonspecific binding was determined with 1 mM NECA. Incubations were carried out in a total volume of 0.2 mL of Tris-HCl buffer, 50 mM, pH 7.4, at room temperature (23 °C) for 60 min. Incubation was terminated by rapid filtration using a Brandel 48-channel cell harvester through GF/B glass fiber filters. Filters were rinsed three times with 2 mL each of ice-cold Tris-HCl buffer, 50 mM, pH 7.4. A_{2B} receptor binding studies were performed twice each in duplicate.

The adenosine A_3 receptor binding assay was carried out at membranes of CHO cells stably transfected with the human A_3-AR using [^{3}H]PSB-11 (1.96 TBq/mmol, Amersham, Germany) as radioligand. Cell culture and membrane preparation procedure was performed as described previously.[61,62] Assays were performed in a total volume of 0.5 mL of Tris-HCl buffer 50 mM, pH 7.4. Nonspecific binding was determined with 100 μM R-PIA. [^{3}H]PSB-11 was used in a final concentration of 0.5 nM. After 45 min, incubation was terminated by rapid filtration using a Brandel 48-channel cell harvester through GF/B glass fiber filters. Filters were rinsed three times with 2 mL each of ice-cold Tris-HCl buffer, 50 mM, pH 7.4. A_3 receptor binding studies were performed twice each in duplicate.

Radioactivity on the punched-out filters was measured after 9 h of preincubation with 2.5 mL of Ultima Gold scintillation cocktail (Canberra Packard, Dreieich, Germany).

GTP Shift Experiments. GTP shift experiments were performed twice each in duplicate. Inhibition of binding of [^{3}H]-DPCPX (4.40 TBq/mmol, Amersham, Germany) by the compound was measured both in the absence and in the presence of 100 μM GTP. The assays were performed under the same conditions as described above for the A_1 assay except for the use of a different radioligand. Nonspecific binding was determined using 10 μM unlabeled DPCPX. Inhibition of binding of [^{3}H]PSB-11 (1.96 TBq/mmol, Amersham, Germany) by the compound was measured both in the absence and in the presence of 100 μM GTP. The assays were carried out under the same conditions as described above for the A_3 assay. Nonspecific binding was determined using 100 μM R-PIA.

Cyclic AMP Accumulation. Culture flasks with confluently grown cells were washed with PBS buffer, and cells were detached with a cell scraper. A membrane preparation of the cells was obtained according to the method described below. The membrane fraction was resuspended in Tris buffer, 50 mM, pH 7.4, supplemented with 4 mM EDTA, 10 mM $MgCl_2$, and 5 μM GTP. The mixture was preincubated for 10 min at 37 °C with 2 IU of adenosine deaminase to remove endogenous adenosine. Compounds dissolved in DMSO were diluted in Tris buffer, 50 mM, pH 7.4, containing EDTA (4 mM), $MgCl_2$ (10 mM), GTP (5 μM), forskolin (10 μM), and ATP (1 mM) and incubated for 10 min at 37 °C with 90 μL of the membrane suspension. Incubation was terminated by rapidly heating to 98 °C for 2 min. After the tubes were cooled to room temperature, the tubes were centrifuged at 2000*g*, 15 min, 4 °C, using an Allegra 21 R centrifuge (Beckman Coulter, Germany). The supernatant (50 μL) was assayed in a charcoal adsorption assay using a commercially available kit (Amersham, Germany) to determine cAMP accumulation. Each experiment was carried out in duplicate up to three times at least.

Cell Culture. CHO cells stably transfected with the human A_3AR (CHO-hA_3) were grown adherently and maintained in Dulbecco's modified Eagles medium F12, supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamate (2 mM) at 37 °C, 5% CO_2. Cells were grown to confluence and subcultured twice a week in a ratio of 1:5. For cAMP assays, the culture medium was removed, cells were washed with PBS, and membrane preparation was done immediately following the procedure described by Klotz et al.[62]

Data Analysis. Data were analyzed using Prism 3.0 (Graph Pad, San Diego, CA). For nonlinear-regression analysis, the Cheng−Prusoff equation and the K_D values of 0.5 nM for [^{3}H]-CCPA, 8 nM for [^{3}H]MSX-2, 50 nM for [^{3}H]PSB-298, 0.28 nM for [^{3}H]DPCPX, and 4.9 nM for [^{3}H]PSB-11 were used to calculate K_i values from the obtained IC_{50} values.

GTP shift experiments were analyzed by using nonlinear regression analysis both in the absence and in the presence of GTP. The GTP shift was calculated by dividing the apparent IC_{50} value of the ligand in the presence of GTP by the apparent IC_{50} value in the absence of GTP.

To perform statistical comparisons between groups, data were subjected to analysis of variance followed by an unpaired *t*-test.

Ester Hydrolysis. Ester hydrolysis of compound **4** was performed with carboxylesterase from porcine liver (EC 3.1.1.1, 1840 IU/mL) obtained from Sigma Aldrich, Germany. A 1 mM stock solution of compound **4** in DMSO (12.5 μL) was diluted with Tris-HCl buffer, 5 or 50 mM, pH 8.0 (concentration of **4** was 25 μM), and an amount of 2 μL of carboxylesterase was added. Incubation was carried out at 25 °C for 60 min. A sample of 12.5 μL of a 1 mM stock solution of compound **4** in DMSO was diluted with Tris-HCl buffer, 50 mM, pH 7.4 (concentration of **4** 25 μM), and incubated with rat brain cortex (protein concentration ca. 70 μg/mL) at room temperature (23 °C) for 90 min.

Incubation was terminated by heating the sample at 99 °C for 10 min. The samples were centrifuged at 10000*g* for 5 min at room temperature (23 °C), and the resulting supernatants were used for subsequent analysis by capillary electrophoresis (CE). The final concentration that was injected was 25 μM compound **4**.

To confirm the degradation by carboxylesterase, an adenosine A_1 receptor binding assay was carried out in the presence of 2 μL of carboxylesterase after preincubation of the diester with the enzyme at 25 °C for 60 min. The assay was performed under the same conditions as described above for the A_1 assay with [^{3}H]CCPA as radioligand. Two different buffer concentrations (5 and 50 mM) were used to dilute compound **4**. CE analyses showed that no differences in the peak areas of the hydrolyzed products could be observed and the buffer concentration had no effect on the enzymatic reaction.

Capillary Electrophoresis. CE separations were carried out using a P/PACE system MDQ glycoprotein (Beckman Coulter Instruments, Fullerton, CA) equipped with a DAD detection system. The electrophoretic separations were carried out using an eCAP fused-silica capillary [40 cm (30 cm effective length) × 75 μm internal diameter (i.d.) × 375 μm outside diameter (o.d.) obtained from Beckman Coulter]. On-line UV detection was performed in the range of 190−350 nm. The runs were performed under the following conditions: T = 25 °C, λ_{max} = 260 nm, current = 95 μA, running buffer 25 mM sodium tetraborate buffer, 100 mM SDS, pH 8.5. The capillary was washed with 0.1 N NaOH for 2 min, deionized water for 1 min, and 25 mM sodium tetraborate buffer, 100 mM SDS, pH 8.5, for 1 min before each injection. Injections were made by applying 0.1 psi of pressure to the sample solution for 25 s.

The CE instrument was fully controlled through a personal computer, which operated with the analysis software 32 KARAT obtained from Beckman Coulter. The evaluation of the electropherograms was done using the same software. The temperature was kept constant at 25 °C, and the samples were also stored at the same temperature.

Chemicals. Tris(hydroxymethyl)aminomethane was obtained from Acros Organics, Geel, Belgium. Sodium tetraborate, boric acid, and sodium dodecyl sulfate (SDS) were obtained from Sigma Aldrich Germany. All other reagents were obtained from Sigma Aldrich, Germany.

Molecular Modeling. The molecular models of the human A_1AR and A_3AR were built using the homology model tool of MOE 2003.02.[63] The X-ray structure of bovine rhodopsin was used as a structural template. The alignment of the seven transmembrane helices is based on the WHAT-IF Profile V2.0 for class A GPCRs, taken from the GPCRDB database (http://www.gpcr.org/7tm/).[64]

The second extracellular loop (EL2) was deleted, and subsequently high-affinity ligands (2-phenyl-8-ethyl-4-methyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-on (PSB-10, antagonist)[65] and N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (IB-MECA, agonist) for the hA_3AR, and N^6-cyclopentyladenosine (CPA, agonist) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, antagonist) for the hA_1AR) were docked using the FLEXI-DOCK software from the Sybyl 6.9 modeling package.[66] These docked ligands were taken into account (as environment atoms) in the building of the final homology model, again using MOE. In this way, it was ensured that the binding pocket was large enough to be occupied by the ligand. The 3D structures were energy-minimized by using the Kollman all-atom force field for up to 1000 steps or until a gradient of 0.05 kcal/(mol·Å) was reached.

Models of adenosine receptor ligands used in this study were constructed using the "Sketch Molecule" module of Sybyl 6.9. The structures were minimized with molecular mechanics (using the Tripos force field, conjugate-gradient minimizing method) and subsequently with the MOPAC software (using the semiempirical AM1 method).[67] Partial atomic charges were also taken from the MOPAC calculation. Docking of the ligands was carried out by the GOLD-Software[68] with standard default settings.

Results from the GOLD-Docking were subsequently minimized using the Minimize Subset option in Sybyl (the ligand and amino acids at a distance of 6 Å were fully minimized, and those within 12 Å were taken into account). Minimization was run up to 1000 steps or until a gradient of 0.05 kcal/(mol· Å) was reached.

Acknowledgment. We thank Dr. W. Beil, Institute for Pharmacology, Medical University of Hannover, for performing the cytotoxicity tests, Dr. P. Janning, MPI for Molecular Physiology, Dortmund, and Dr. G. Eckhardt, Institute for Chemistry, University of Bonn, for making all MS measurements. Financial support from the Deutsche Forschungsgemeinschaft (Grant Ko-902/2-4), the Fonds der Chemischen Industrie (Grants G.M.K. No. 164 357, C.E.M. No. 160 310), and the Bundesministerium für Bildung und Forschung (BMBF) is gratefully acknowledged. S.G. was supported by a scholarship from the Deutsche Forschungsgemeinschaft within the Graduiertenkolleg GRK 804.

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JM031092G