2′-*C*-Methyl Analogues of Selective Adenosine Receptor Agonists: Synthesis and Binding Studies†

Palmarisa Franchetti,* Loredana Cappellacci, Stefano Marchetti, Letizia Trincavelli,‡ Claudia Martini,‡ Maria R. Mazzoni,‡ Antonio Lucacchini,‡ and Mario Grifantini*

Dipartimento di Scienze Chimiche, Università di Camerino, Via S. Agostino 1, 62032 Camerino, Italy, and Istituto Policattedra di Discipline Biologiche, Università di Pisa, Via Bonanno 6, 56126 Pisa, Italy

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2′-*C*-Methyl analogues of selective adenosine receptor agonists such as (*R*)-PIA, CPA, CCPA, NECA, and IB-MECA were synthesized in order to further investigate the subdomain that binds the ribose moiety. Binding affinities of these new compounds at A_1 and A_{2A} receptors in bovine brain membranes and at A_3 in rat testis membranes were determined and compared. It was found that the 2′-*C*-methyl modification resulted in a decrease of the affinity, particularly at A_{2A} and A_3 receptors. When such modification was combined with N^6-substitutions with groups which induce high potency and selectivity at A_1 receptors, the high affinity was retained and the selectivity was increased. Thus, 2-chloro-2′-*C*-methyl-N^6-cyclopentyladenosine (2′-Me-CCPA), which displayed a K_i value of 1.8 nM at A_1 receptors, was selective for A_1 vs A_{2A} and A_3 receptors by 2166- and 2777-fold, respectively, resulting in one of the most potent and A_1-selective agonists so far known. In functional assay, this compound inhibited forskolin-stimulated adenylyl cyclase activity with an IC_{50} value of 13.1 nM, acting as a full agonist.

Adenosine affects a wide variety of physiological functions by acting on central nervous, cardiovascular, immune, and hormonal systems; adenosine is also able to inhibit lipolysis, platelet aggregation, and neurotransmitter release from nerve endings and to potentiate histamine release from mast cells.[1−4] The adenosine actions are mediated by membrane receptors coupled to guanyl nucleotide-binding proteins (G-proteins). Four major subtypes of adenosine receptors have been so far identified by pharmacological and molecular biological techniques: A_1, A_{2A}, A_{2B}, and A_3. These receptors have been cloned from several species including human.[5] A_1 and A_3 receptors are coupled to a variety of second-messenger systems, including inhibition of adenylyl cyclase, inhibition or stimulation of phosphoinositol turnover, activation of guanylyl cyclase, and, in the case of A_1 receptors, activation of potassium channels and inhibition of calcium influx, while activation of A_{2A} and A_{2B} receptors stimulates adenylyl cyclase.[1,2,6]

Highly selective ligands as agonists/antagonists for adenosine receptors have been developed. However, till now, all known adenosine agonists are closely related to the chemical structure of adenosine itself. Substitution at N^6 or C2 may enhance affinity and may impart $A_1/A_2/A_3$ selectivity.[2,7,8] Other modifications of the adenine moiety usually lead to inactive or, few, active compounds.[8]

The ribose recognition domain appears to be of considerable importance for activity at adenosine receptors, and 2′-, 3′-, and 5′-hydroxy groups contribute markedly to the potency of adenosine analogues as agonists. Replacement of the $-CH_2OH$ group of adenosine or adenosine analogues with an *N*-alkyl 5′-carboxamide group (5′-uronamide modification) results in potent agonists. The highest agonist activity of the adenosine-5′-carboxamides is shown by those with small alkyl groups or a cyclopropyl group. Depending on the substituents at N^6 or C2 positions of the adenine moiety, the adenosine-5′-uronamide analogues have been reported to enhance the affinity as agonists at A_{2A} or A_3 adenosine receptors.[2,6,8] The 4′-*C*-methyl substitution of adenosine was poorly tolerated, yet when combined with other favorable modifications, such as the 4′-*C*-methyl derivative of N^6-(benzylamino)adenosine-5′-(*N*-methyluronamide), the potency and selectivity at A_3 receptors may be maintained.[8] The 3′-*C*-methyl analogue of adenosine was found to bind weakly but with A_1 selectivity.[8]

To further investigate the subdomain that binds the ribose moiety, we studied a series of 2′-*C*-methyl derivatives of A_1-, A_{2A}-, and A_3-selective adenosine receptor agonists. In particular, the 2′-*C*-methyl analogues of N^6-cyclopentyladenosine (CPA), 2-chloro-N^6-cyclopentyladenosine (CCPA), adenosine-5′-ethyluronamide (NECA), and N^6-(3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine (IB-MECA) have been synthesized and their affinity for A_1, A_{2A}, and A_3 adenosine receptors determined by binding studies.

Chemistry

2′-*C*-Methyladenosine (**5**, 2′-Me-Ado) and N^6-substituted derivatives **6**−**9** were synthesized as shown in

† Abbreviations: ATP, adenosine 5′-triphosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; CGS 21680, 2-[4-(2-carboxyethyl)phenyl]-ethylamino-5′−(*N*-ethylcarbamoyl)adenosine; CHA, N^6-(cyclohexyl)-adenosine; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DTT, dithiothreitol; GTP, guanosine 5′-triphosphate; PMSF, phenylmethanesulfonyl fluoride; (*R*)-PIA, N^6-[(*R*)-phenylisopropyl]adenosine.

* To whom correspondence should be addressed at Università di Camerino. Tel: (+39)737-402233. Fax: (+39)737-637345. E-mail: grima@camserv.unicam.it.

‡ Università di Pisa.

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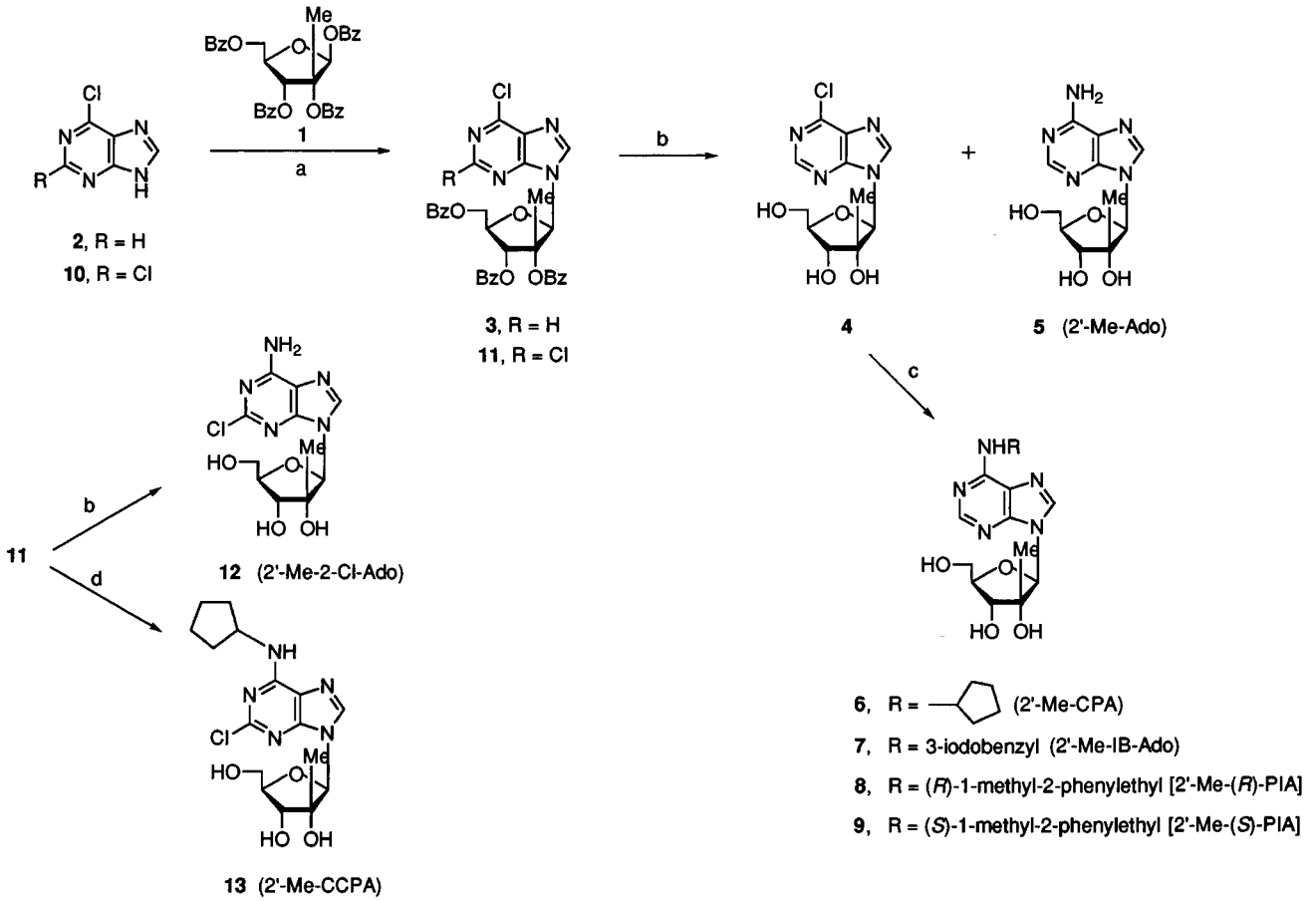
Scheme 1[a]



[a] Reagents: (a) Me_3SiOTf/CH_3CN, DBU; (b) NH_3/MeOH; (c) RNH_2, EtOH, heat; (d) cyclopentylamine, EtOH, reflux.

Scheme 1. Coupling of 1,2,3,5-tetrabenzoyl-2-*C*-methyl-β-D-ribofuranose[9] (**1**) and 6-chloropurine (**2**) with trimethylsilyl trifluoromethanesulfonate in acetonitrile in the presence of DBU gave the nucleoside intermediate **3** which was debenzoylated with methanolic ammonia to afford 6-chloro-2′-*C*-methylpurine riboside (**4**) and 6-amino-2′-*C*-methylpurine riboside (**5**, 2′-Me-Ado). Nucleophilic displacement of the chlorine atom of **4** with cyclopentylamine, 3-iodobenzylamine, *l*-amphetamine, and *d*-amphetamine gave compounds **6**−**9**. 2′-Me-2-Cl-Ado (**12**) and 2′-Me-CCPA (**13**) were prepared in a similar way starting from **1** and 2,6-dichloropurine (**10**).

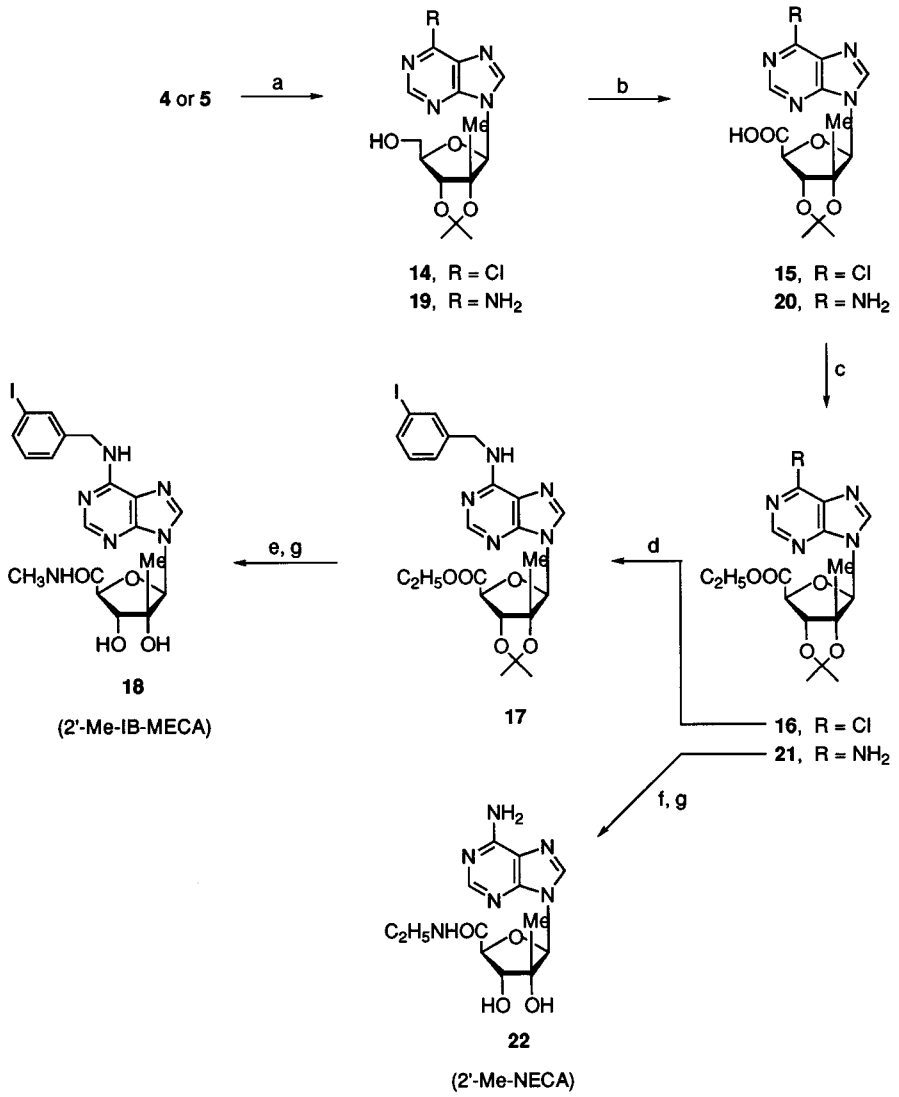
2′-*C*-Methyl derivative of IB-MECA was prepared as depicted in Scheme 2. Oxidation of the isopropylidene derivative of **4** (**14**) with 2 M chromium trioxide in aqueous H_2SO_4 (3 M) gave uronic acid **15**. Compound **15** was converted into ethyl ester **16** by reaction with $SOCl_2$ and anhydrous ethanol. Displacement of the 6-Cl atom of **16** with 3-iodobenzylamine gave the intermediate **17** which was converted in the 2′-Me-IB-MECA (**18**) by reaction with methylamine, followed by deacetalation with 1 N HCl.

The 2′-*C*-methyl derivative of NECA was more conveniently prepared by oxidation of the 2′,3′-isopropylidene derivative of **5** (compound **19**) with $KMnO_4$/KOH to give the uronic acid **20**. Ethyl ester **21**, obtained from **20** with $SOCl_2$ and anhydrous ethanol, was converted into 2′-Me-NECA (**22**) by reaction with ethylamine.

Information concerning the predominant solution conformation of these 2′-*C*-methylribonucleosides was obtained via ^1H NMR experiments. Nuclear Overhauser enhancement (NOE) effects in compounds **5**−**9**, **12**, and **13** were determined in DMSO-d_6 + D_2O (5:1). The complete lack of H-8 enhancement when H-1′ was irradiated supports a spatial arrangement where H-8 and H-1′ are not proximate, as would be the case in the anti conformer. The anti conformation was confirmed by the observation of NOE effects on $O_{5'}$-H and on H-3′ when H-8 was irradiated. On the contrary, when H-1′ was irradiated, an enhancement of H-8 was observed in the case of uronamides **18** and **22**, indicating that a population of the syn conformation is present in these compounds. However, because the H-8 enhancement in **18** and **22** (2.1%) is lower than that observed in NECA (22%),[10] the conformations of 2′-Me-NECA and 2′-Me-IB-MECA should diverge slightly from those of the nonmethylated analogues. On the basis of correlations reported by Rosemeyer et al.,[11] the observed H-1′ enhancement translates into a 19% population of syn conformers.

Further information concerning the solution conformation was obtained by the coupling constant values. The 2′-C substitution precludes getting information from $J_{1'2'}$ and $J_{2'3'}$ values leaving only $J_{3'4'}$ as a clue to the sugar puckering. It was found that in compounds **5**−**9**, **12**, and **13** the $J_{3'4'}$ value is 7.7 Hz, indicating that these nucleosides are predominantly N-puckered. An N-puckered conformation was present also in the uronamides **18** and **22** as revealed from the $J_{3'4'}$ value.

Scheme 2[a]



[a] Reagents: (a) $(CH_3)_2CO$, $CH(OC_2H_5)_3$ [or $(CH_3)_2C(OCH_3)_2$], TsOH; (b) Cr_2O_3 (2 M) in aqueous H_2SO_4 (3 M), or $KMnO_4$, KOH; (c) $SOCl_2$, EtOH; (d) 3-I-$PhCH_2NH_2$·HCl, Et_3N, EtOH, reflux; (e) CH_3NH_2, −20 °C to room temperature; (f) $C_2H_5NH_2$, −20 °C to room temperature; (g) 1 N HCl.

Results

The new 2′-*C*-methyl derivatives were tested in radioligand binding assays to determine their affinities toward A_1, A_{2A}, and A_3 adenosine receptors (Table 1). Affinities for A_1 and A_{2A} receptors were determined in competition assays of [^{3}H]CHA and [^{3}H]CGS 21680 to bovine cortical (A_1) and striatal (A_{2A}) membranes, respectively.[12] Rat cortical (A_1) and striatal (A_{2A}) membranes were analyzed with similar results. Affinity for A_3 receptors was determined in competition assays of [^{3}H]-(*R*)-PIA to rat testis membranes in the presence of the A_1-selective antagonist DPCPX.[13] Under these experimental conditions, specific binding of [^{3}H]-(*R*)-PIA was saturable and data were fitted by a one-site model. The K_d and B_{max} values were 36 nM and 707 fmol/mg of protein, respectively. This low-affinity binding site showed a rank order of potency for agonists (CHA ≥ NECA = (*R*)-PIA > (*S*)-PIA) similar to that described for rat A_3 adenosine receptor expressed in CHO cells.[14] The potency of the new 2′-*C*-methyl derivatives was compared with that of nonmethylated analogues and 3′-*C*-methyladenosine.[15]

3′-*C*-Methyladenosine bound weakly with A_1 selectivity. This result confirms the binding data reported by Siddiqi et al.[8] determined in rat cortical (A_1) and striatal (A_{2A}) membranes and with A_3 receptor expressed in CHO cells. 2′-*C*-Methyladenosine was much more potent than 3′-*C*-methyladenosine at both A_1 and A_{2A} receptors, while the two isomers were substantially inactive at A_3 receptors.

So, the introduction of a methyl group in the ribose moiety of adenosine is better tolerated at the 2′- than 3′-position, in particular in the case of A_1 receptors. 2′-*C*-Methyl-substituted analogues of the A_1-selective agonists CPA, (*R*)-PIA, and CCPA were slightly less potent at A_1 receptors (K_i values in the low-nanomolar range) than the parent compounds (K_i values in the subnanomolar range), but their affinities for A_{2A} and A_3 receptors were very low. Thus, the 2′-*C*-methyl modification may serve as a means of increasing A_1 selectivity in general. The most potent compound at A_1 receptors proved to be 2′-Me-CCPA (K_i value of 1.8 nM) which was 2166-fold selective for A_1 vs A_{2A} and 2777-

Table 1. Affinity of 2′-*C*-Methyladenosine Derivatives in Radioligand Binding Assays at Bovine Brain A_1, A_{2A}, and Rat Testis A_3 Receptors[a–c]

	K_i (nM) or % inhibition[d]			selectivity		
compd	A_1[a]	A_{2A}[b]	A_3[c]	A_{2A}/A_1	A_3/A_1	A_3/A_{2a}
5, 2′-Me-Ado	740 ± 60	3420 ± 750	0%	4.6	>13.5	>2.9
6, 2′-Me-CPA	9.5 ± 0.8	7220 ± 930	208 ± 38	760	21.9	0.028
7, 2′-Me-IB-Ado	20.1 ± 3	935 ± 87	3250 ± 700	46.5	162	3.5
8, 2′-Me-(*R*)-PIA	4.7 ± 0.6	7440 ± 1100	1082 ± 260	1582	230	0.14
9, 2′-Me-(*S*)-PIA	50 ± 6.2	17%	10000 ± 2500	>200	200	<1
12, 2′-Me-Cl-Ado	533 ± 65	1330 ± 320	10%	2.5	>18.8	>7.5
13, 2′-Me-CCPA	1.8 ± 0.4	3.900 ± 950	5000 ± 825	2166	2777	1.28
18, 2′-Me-IB-MECA	254 ± 41	1533 ± 112	320 ± 72	6.0	1.2	0.2
22, 2′-Me-NECA	142 ± 35	218 ± 48	550 ± 92	1.5	3.8	2.5
2-Cl-Ado	20.2 ± 4.2	120 ± 15	3200 ± 800	5.9	158.4	26.6
3′-Me-Ado	49500 ± 8300	12%	8%	>202	>202	
CPA	0.3 ± 0.15	385 ± 80	26 ± 7	1327	89	0.06
CCPA	0.35 ± 0.13	580 ± 75	30 ± 6	1657	86.6	0.05
(*R*)-PIA	0.6 ± 0.2	750 ± 85	53 ± 4[e]	1263	88	0.07
(*S*)-PIA	11.7 ± 3.7	1800 ± 570	241 ± 12[e]	154	13	0.13
NECA	14 ± 4.2	16 ± 3.1	49 ± 3[e]	1.1	3.5	3.06
IB-Ado	18.6 ± 3.7	20.4 ± 5.	10.2 ± 2	1.09	0.54	0.5
IB-MECA	33.1 ± 5.7	116 ± 23	30 ± 7	3.51	0.9	0.25

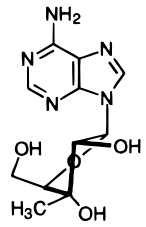
[a] Inhibition of specific [^{3}H]CHA binding to bovine brain cortical membranes expressed as K_i ± SEM in nM (n = 3). [b] Inhibition of specific [^{3}H]CGS 21680 binding to bovine striatal membranes expressed as K_i ± SEM in nM (n = 3). [c] Inhibition of specific [^{3}H]-(*R*)-PIA binding to rat testis membranes in the presence of 150 nM DPCPX expressed as K_i ± SEM in nM (n = 3). [d] A percent value indicates the percent inhibition of specific binding at a concentration of 10 μM. [e] Reference 13. Each value represents the average of three experiments.

fold for A_1 vs A_3. So, this compound is one of the most A_1-selective agonists reported so far.

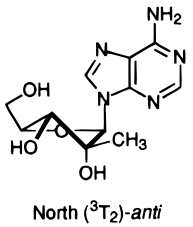
The stereoselectivity characteristic of the N^6 region of A_1, A_{2A}, and A_3 receptors is maintained in the 2′-*C*-methyl analogues. In fact, 2′-Me-(*R*)-PIA is more potent than 2′-Me-(*S*)-PIA at all three adenosine receptor subtypes.

Introduction of the chloro function in position 2 of 2′-Me-Ado resulted in a slight increase of affinity at A_1 and A_{2A} receptors. The 2′-methyl modification in NECA and IB-MECA was not well-tolerated, in particular at A_{2A} and A_3 receptors.

Some considerations should be made about the influence of the conformation of the 2′-*C*-methyl-substituted adenosine analogues on the affinity for adenosine receptors. It was reported that 3′-*C*-methyladenosine was resistant to adenosine deaminase (ADA), while 2′-Me-Ado was deaminated by ADA, even though the rate of deamination was 1/25 that observed with adenosine as substrate.[16] The different affinity of 3′-*C*-methyl- and 2′-*C*-methyladenosine for ADA was ascribed to the different conformations of the furanose ring of these nucleosides in solution. In fact, the introduction of a methyl group in the 3′- and 2′-positions of adenosine brings about an increase of the conformational rigidity of the furanose ring. ^{1}H NMR data obtained by us and other authors[17] showed that in solution 3′-*C*-methyladenosine has a marked preference for the south ($^2T_3$)-syn conformation, while the conformation of 2′-*C*-methyladenosine is predominantly north ($^3T_2$)-anti:



South ($^2T_3$)-*syn*



North ($^3T_2$)-*anti*

Thus, the high affinity of 2′-Me-Ado and its N^6-substituted analogues at A_1 adenosine receptors, compared to the very low affinity of 3′-*C*-methyladenosine, might be related to the particular conformation, confirming the hypothesis that adenosine and adenosine derivatives which are A_1-selective agonists bind the receptor in the anti conformation, probably with the furanose ring in the N-puckered conformation.[18]

The most potent and A_1-selective compounds were also tested for ability to inhibit forskolin-stimulated adenylyl cyclase in rat cortical membranes. The efficacy of these compounds was compared with that obtained for CHA and CPA (Figure 1). In our assays, CHA was a more potent inhibitor (IC_{50} 1.4 ± 0.7 nM) of adenylyl cyclase activity than CPA (IC_{50} 11.6 ± 0.7 nM), while the maximal efficacy was similar for both agonists (Table 2). The maximal inhibition for the new derivatives ranged from 11% to 15%. Compound **8** was the most active inhibitor (IC_{50} 1.6 ± 0.7 nM; maximal inhibition, 13.7%) (Figure 1A and Table 2), while the dose–response curves for **6** and **7** were shallow and the maximal effects were modest (approximately 11%). Finally, 2′-Me-CCPA (**13**) showed a dose–response curve and a IC_{50} value similar to those for CPA (Figure 1A and Table 2). A discrepancy between IC_{50} values in the functional assay and K_i values in binding assay was observed for **13** and CPA. In fact, the K_i values of these compounds were lower by a factor of 7 and 39, respectively.

To rule out the possible partial agonist properties of **13**, we examined the effects of CHA, a potent and selective A_1 agonist, on forskolin-stimulated adenylyl cyclase activity in the presence and absence of **13** (30 nM) (Table 3). There was no significant right shift of the inhibition curve in the presence of the compound (IC_{50} 1.6 ± 0.9 nM), thus no antagonist properties of 2′-Me-CCPA could be detected. Therefore, this compound behaved as a full agonist.

Since 2′-Me-IB-Ado and 2′-Me-CPA do not reach the maximal inhibition of adenylyl cyclase, their activity as

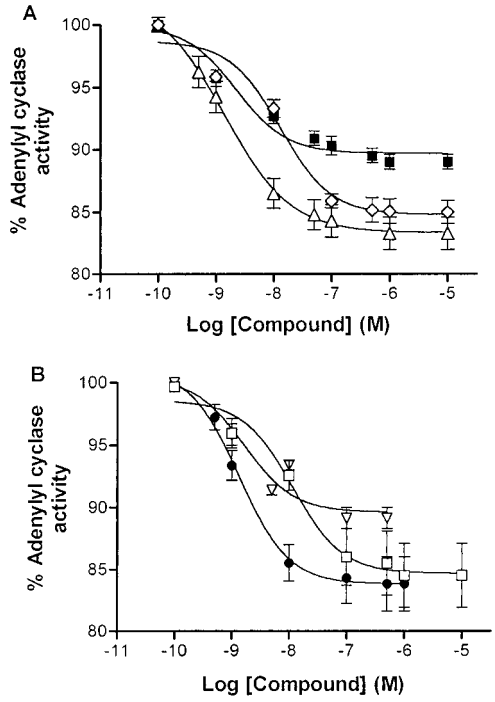


Figure 1. Inhibition of adenylyl cyclase in rat cortical membranes. The assay was carried out as described in Biological Methods in the presence of 100 μM forskolin. Each data point is shown as mean ±SEM of at least three independent experiments. Concentration-dependent effects on adenylyl cyclase by (panel A) 2′-Me-CPA (■), 2′-Me-(*R*)-PIA (△), 2′-Me-CCPA (◇) and (panel B) CHA (●), CPA (□), and 2′-Me-IB-Ado (▽).

Table 2. Inhibition of Adenylyl Cyclase Activity in Rat Cortical Membranes by 2′-*C*-Methyladenosine Derivatives[a]

compd	IC_{50} (nM)	% maximal inhibition
CHA	1.4 ± 0.7	16.2 ± 2.2
CPA	11.6 ± 0.7	15.5 ± 2.6
2′-Me-CPA	2.3 ± 0.6	11.0 ± 0.6
2′-Me-(*R*)-PIA	1.6 ± 0.7	13.7 ± 1.7
2′-Me-IB-Ado	1.6 ± 0.4	10.9 ± 0.8
2′-Me-CCPA	13.1 ± 0.6	15.1 ± 1.6

[a] IC_{50} values were obtained from nonlinear curve fitting of data using GraphPad computer program. The maximal inhibitory effects were at 10 μM. All values are the mean ± SEM of three independent experiments.

Table 3. Effect of Derivatives **6**, **7**, and **13** on CHA Inhibition of Adenylyl Cyclase Activity in Rat Cortical Membranes[a]

compd	IC_{50} (nM)	% maximal inhibition
CHA	1.4 ± 0.7	16.2 ± 2.2
CHA + 2′-Me-CCPA	1.6 ± 0.9	16.1 ± 0.8
CHA + 2′-Me-IB-Ado	1.5 ± 0.8	16.0 ± 0.9
CHA + 2′-Me-CPA	1.4 ± 0.6	16.2 ± 0.9

[a] Inhibition of adenylyl cyclase by CHA in the absence and presence of 2′-Me-CCPA (30 nM), 2′-Me-IB-Ado (30 nM), and 2′-Me-CPA (50 nM). IC_{50} values were obtained from nonlinear curve fitting of data using GraphPad computer program. All values are the mean ± SEM of three independent experirnents.

partial agonists can be suspected. To verify whether these compounds behave as partial agonists for the rat A_1 adenosine receptor, we evaluated their effects on CHA dose−response curves of adenylyl cyclase inhibition. The results showed no significant right shift of the inhibition curve in the presence of either 30 nM 2′-Me-IB-Ado (IC_{50} 1.5 ± 0.8 nM) or 50 nM 2′-Me-CPA (IC_{50} 1.4 ± 0.6 nM). Therefore both compounds showed full agonist properties (Table 3). Moreover we cannot exclude that at high concentrations (1−10 μM) some compounds with a low A_{2A}/A_1 ratio also bind to A_{2A} receptors and thus determine a receptor-mediated activation of adenylyl cyclase. In this case, the dose−response curves cannot reach maximal effects as those obtained for CHA and CPA. Comparing the ability of 2′-Me-IB-Ado and 2′-Me-CPA to inhibit agonist binding to bovine A_1 and A_{2A} adenosine receptors is evident that these compounds have a lower A_{2A}/A_1 ratio than CPA and 2′-Me-CCPA.

Conclusions

In conclusion, it was found that the 2′-*C*-methyl modification in adenosine analogues, which are selective agonists for adenosine receptors, resulted in a decrease of the affinity, particularly at A_{2A} and A_3 receptors. When such a modification was combined with N^6-substitutions with groups which induce high potency and selectivity at A_1 receptors, the high affinity was retained and the selectivity increased. The high affinity and selectivity at A_1 receptors of 2′-*C*-methylribosyl analogues of adenosine may be related to their preferential conformation in solution which was determined to be anti with a north ($^3T_2$) puckered furanose ring form.

The 2-chloro-2′-methyl-N^6-cyclopentyladenosine (2′-Me-CCPA), the most potent and selective compound at A_1 receptors among the 2′-methyl derivatives of adenosine analogues considered in this study (K_i value of 1.8 nM, 2166-fold selective for A_1 vs A_{2A} and 2777-fold for A_1 vs A_3), is one of the most potent and A_1-selective agonists so far known. However, its intrinsic activity as an inhibitor of adenylyl cyclase is approximately 10-fold lower than that found for CHA. Similarly, for rat A_{2A} adenosine receptors CGS 21680, a potent and selective agonist, is less effective in stimulating adenylyl cyclase than NECA, a nonselective agonist.[19] In any case, 2′-Me-CCPA is potentially useful as a pharmacological probe to define more clearly the physiological role, distribution, and regulation of A_1 adenosine receptors.

Experimental Section

Chemistry. Melting points were determined on a Büchi apparatus and are uncorrected. Elemental analyses were determined on a Carlo Erba model 1106 analyzer. Thin layer chromatography (TLC) was carried out on silica gel 60 F-254 plates, and silica gel 60 Merck (70−230 and 230−400 mesh) for column chromatography was used. Nuclear magnetic resonance ^{1}H NMR spectra were determined at 300 MHz, with a Varian VXR-300 spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard. All exchangeable protons were confirmed by addition of D_2O. Stationary NOE experiments were run on degassed solutions at 25 °C. A presaturation delay of 1 s was used, during which the decoupler low power was set at 20 dB attenuation.

6-Chloro-9*H*-(2-*C*-methyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)purine (3). To a stirred mixture of 2′-*C*-methyl-1,2,3,5-tetra-*O*-benzoyl-D-ribose[9] (4.3 g, 7.41 mmol), 6-chloropurine (1.25 g, 8.08 mmol), and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (3.31 mL, 22.23 mmol) in anhydrous acetonitrile (30 mL) cooled at 0 °C under a nitrogen atmosphere was slowly added Me_3SiOTf (5.72 mL, 29.64 mmol). The resulting mixture was heated at 60 °C for 4 h, cooled to room temperature, and shaken between $NaHCO_3$ (1 M) and CH_2Cl_2. The organic layer was separated and dried over Na_2SO_4. The solvent was

evaporated to dryness, and the crude residue was purified by flash chromatography on silica gel (*n*-hexanes−EtOAc, 88:12) to provide **3** as a white solid (3.77 g, 83% yield): mp 128−130 °C. TLC (*n*-hexanes−EtOAc, 80:20): R_f = 0.28. ^{1}H NMR (DMSO-d_6): δ 1.60 (s, 3H, CH_3), 4.85 (m, 3H, H-4′, H-5′), 6.08 (d, J = 4.9 Hz, 1H, H-3′), 6.95 (s, 1H, H-1′), 7.4−7.73 (2m, 9H, Ph), 7.90−8.10 (m, 6H, Ph), 8.90 (s, 1H, H-2), 9.02 (s, 1H, H-8). Anal. ($C_{32}H_{25}ClN_4O_7$) C, H, N.

6-Chloro-9*H*-(2-*C*-methyl-β-D-ribofuranosyl)purine (4) and 9*H*-(2-*C*-Methyl-β-d-ribofuranosyl)adenine (5). Compound **3** (2 g, 3.26 mmol) was treated with methanolic ammonia (40 mL, saturated at 0 °C) and stirred at room temperature for 7 h in a pressure bottle. The solvent was evaporated to dryness, and the solid residue was purified by flash chromatogaphy on silica gel ($CHCl_3$−MeOH, 97:3) to yield **4** as a white solid (72%): mp 200−202 °C dec. TLC ($CHCl_3$−MeOH, 90:10): R_f = 0.41. ^{1}H NMR (DMSO-d_6): δ 0.80 (s, 3H, CH_3), 3.76 (dd, J = 3.0, 4.9 Hz, 1H, H-5′), 3.85 (dd, J = 1.9, 4.8 Hz, 1H, H-5′), 4.0 (t, J = 2.3 Hz, 1H, H-4′), 4.07 (d, J = 6.7 Hz, 1H, H-3′), 5.3 (d, J = 6.4 Hz, 2H, OH), 5.43 (s, 1H, OH), 6.1 (s, 1H, H-1′), 8.83 (s, 1H, H-2), 9.10 (s, 1H, H-8). Anal. ($C_{11}H_{13}ClN_4O_4$) C, H, N.

Evaporation of the following fraction afforded 8% of **5** as a white solid: mp >230 °C (lit.[19] mp 257−258 °C). TLC ($CHCl_3$-MeOH, 85:15): R_f = 0.26. ^{1}H NMR (DMSO-d_6): δ 0.77 (s, 3H, CH_3), 3.72 (m, 1H, H-5′), 3.82 (m, 1H, H-5′), 3.93 (br s, 1H, H-4′), 4.05 (pseudo t, 1H, H-3′), 5.20 (s, t, d, 3H, OH), 5.95 (s, 1H, H-1′), 7.3 (br s, 2H, NH_2), 8.15 (s, 1H, H-2), 8.48 (s, 1H, H-8).

***N*^{6}-Cyclopentyl-9*H*-(2-*C*-methyl-β-D-ribofuranosyl)-adenine (6).** A mixture of **4** (80 mg, 0.26 mmol) in anhydrous EtOH (2 mL) and cyclopentylamine (0.15 mL, 1.54 mmol) under a nitrogen atmosphere was heated under reflux for 5 h. The mixture was concentrated in vacuo, and the residue was chromatographed on a silica gel column ($CHCl_3$−MeOH, 95:5) to give **6** as a white solid (55 mg, 60%): mp 118−120 °C dec. TLC ($CHCl_3$−MeOH, 90:10): R_f = 0.56. ^{1}H NMR (DMSO-d_6): δ 0.78 (s, 3H, CH_3), 1.53−1.80 (m, 6H, cyclopentyl), 1.95 (m, 2H, cyclopentyl), 3.69 (2m, 1H, H-5′), 3.82, 3.88 (2m, 1H, H-5′), 3.94 (pseudo t, 1H, H-4′), 4.06 (dd, J = 7.0, 8.9 Hz, 1H, H-3′), 4.58 (br s, 1H, NHC*H*), 5.26 (2d, t, 3H, OH), 5.98 (s, 1H, H-1′), 7.73 (d, J = 7.7 Hz, 1H, NH), 8.2 (s, 1H, H-2), 8.48 (s, 1H, H-8). Anal. ($C_{16}H_{23}N_5O_4$) C, H, N.

***N*^{6}-(3-Iodobenzyl)-9*H*-(2-*C*-methyl-β-D-ribofuranosyl)-adenine (7).** A stirred solution of **4** (100 mg, 0.32 mmol) in anhydrous EtOH (4 mL) was treated with 3-iodobenzylamine hydrochloride (96 mg, 0.34 mmol) and triethylamine (0.14 mL, 0.102 mmol), and the mixture was heated at 80 °C for 12 h under nitrogen atmosphere. After cooling to room temperature, the solvent was evaporated to dryness, and the solid residue was purified by chromatography on a silica gel column ($CHCl_3$−MeOH, 98:2) to yield **7** as a white solid (142 mg, 90%): mp 183−185 °C. TLC ($CHCl_3$−MeOH, 95:5): R_f = 0.12. ^{1}H NMR (DMSO-d_6): δ 0.80 (s, 3H, CH_3), 3.71 (2m, 1H, H-5′), 3.83, 3.90 (2m, 1H, H-5′), 3.95 (pseudo t, 1H, H-4′), 4.05 (pseudo t, 1H, H-3′), 4.66 (br s, 2H, NHCH_2), 5.2 (2d, t, 3H, OH), 5.98 (s, 1H, H-1′), 7.1 (t, J = 7.8 Hz, 1H, Ph), 7.38, 7.58 (2d, J = 7.8 Hz, 2H, Ph), 7.75 (s, 1H, Ph), 8.2 (s, 1H, H-2), 8.44 (br s, 1H, NH), 8.5 (s, 1H, H-8). Anal. ($C_{18}H_{20}IN_5O_4$) C, H, N.

***N*^{6}-[(*R*)-Phenylisopropyl]-9*H*-(2-*C*-methyl-β-D-ribofuranosyl)adenine (8).** Compound **8** was obtained from **4** (0.2 mmol) with *d*-amphetamine as described for **6**, as a white solid (60%): mp 125−130 °C dec. TLC ($CHCl_3$−MeOH, 95:5): R_f = 0.29. ^{1}H NMR (DMSO-d_6): δ 0.78 (s, 3H, CH_3), 1.2 (d, J = 6.2 Hz, 3H, CHCH_3), 2.75 (dd, J = 6.9, 13.6 Hz, 1H, CH_2Ph), 3.05 (dd, J = 7.0, 13.5 Hz, 1H, CH_2Ph), 3.7 (2m, 1H, H-5′), 3.82, 3.88 (2m, 1H, H-5′), 3.93 (pseudo t, 1H, H-4′), 4.07 (pseudo t, 1H, H-3′), 4.58 (br s, 1H, C*H*CH_3), 5.22 (s, d, t, 3H, OH), 5.95 (s, 1H, H-1′), 7.2 (2m, 5H, Ph), 7.71 (d, J = 9.1 Hz, 1H, NH), 8.2 (s, 1H, H-2), 8.48 (s, 1H, H-8). Anal. ($C_{20}H_{25}N_5O_4$) C, H, N.

***N*^{6}-[(*S*)-Phenylisopropyl]-9*H*-(2-*C*-methyl-β-D-ribofuranosyl)adenine (9).** Compound **9** was obtained from **4** (0.2 mmol) with *l*-amphetamine as described above, as a white solid (60%): mp 125−130 °C dec. TLC ($CHCl_3$−MeOH, 90:10): R_f = 0.5. ^{1}H NMR (DMSO-d_6): δ 0.78 (s, 3H, CH_3), 1.2 (d, J = 6.2 Hz, 3H, CHCH_3), 2.75 (dd, J = 6.9, 13.6 Hz, 1H, CH_2Ph), 3.05 (q, J = 6.0 Hz, 1H, CH_2Ph), 3.7 (2m, 1H, H-5′), 3.82, 3.88 (2m, 1H, H-5′), 3.92 (pseudo t, 1H, H-4′), 4.08 (m, 1H, H-3′), 4.58 (br s, 1H, C*H*CH_3), 5.22 (2d, t, 3H, OH), 5.95 (s, 1H, H-1′), 7.2 (m, 5H, Ph), 7.71 (d, J = 9.1 Hz, 1H, NH), 8.2 (s, 1H, H-2), 8.48 (s, 1H, H-8). Anal. ($C_{20}H_{25}N_5O_4$) C, H, N.

2,6-Dichloro-9*H*-(2-*C*-methyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)purine (11). The title compound was prepared from 2,6-dichloropurine (**10**) (300 mg, 1.59 mmol) as described for **3** (reaction time 1.5 h). The residue obtained was treated with MeOH to give **11** as a white solid (870 mg, 92%): mp 125−127 °C. TLC (*n*-hexanes−EtOAc, 85:15): R_f = 0.15. ^{1}H NMR (DMSO-d_6): δ 1.6 (s, 3H, CH_3), 4.82 (br s, 3H, H-4′, H-5′), 5.9 (br s, 1H, H-3′), 6.83 (s, 1H, H-1′), 7.37 (t, J = 7.5, 2H, Ph), 7.55, 7.68 (2m, 7H, Ph), 7.86 (d, J = 7.4, 2H, Ph), 8.02−8.15 (dd, J = 7.2, 15.9 Hz, 4H, Ph), 9.0 (s, 1H, H-8). Anal. ($C_{32}H_{24}Cl_2N_4O_7$) C, H, N.

2-Chloro-9*H*-(2-*C*-methyl-β-D-ribofuranosyl)adenine (12). Compound **12** was obtained as described for **5** (white solid, 35%): mp 130−132 °C dec. TLC ($CHCl_3$−MeOH, 90:10): R_f = 0.25. ^{1}H NMR (DMSO-d_6): δ 0.82 (s, 3H, CH_3), 3.72 (2m, 1H, H-5′), 3.82, 3.88 (2m, 1H, H-5′), 3.93 (pseudo t, 1H, H-4′), 4.04 (pseudo t, 1H, H-3′), 5.18 (d, t, 2H, OH), 5.3 (s, 1H, OH), 5.85 (s, 1H, H-1′), 7.82 (br s, 2H, NH_2), 8.50 (s, 1H, H-8). Anal. ($C_{11}H_{14}ClN_5O_4$) C, H, N.

***N*^{6}-Cyclopentyl-2-chloro-9*H*-(2-*C*-methyl-β-D-ribofuranosyl)adenine (13).** The title compound was prepared from **11** (0.46 mmol) in anhydrous MeOH (10 mL) and cyclopentylamine (5.9 mmol) as described for **6** (reaction time 4 h), as a white solid (35%): mp 128−132 °C dec. TLC ($CHCl_3$−MeOH, 95:5): R_f = 0.34. ^{1}H NMR (DMSO-d_6): δ 0.8 (s, 3H, CH_3), 1.5−1.8 (m, 6H, cyclopentyl), 1.95 (m, 2H, cyclopentyl), 3.69 (2m, 1H, H-5′), 3.82, 3.88 (2m, 1H, H-5′), 3.95 (pseudo t, 1H, H-4′), 4.05 (m, 1H, H-3′), 4.42 (m, 1H, NHC*H*), 5.2 (d, t, 2H, OH), 5.4 (br s, 1H, OH), 5.85 (s, 1H, H-1′), 8.32 (d, J = 7.2 Hz, 1H, NH), 8.52 (s, 1H, H-8). Anal. ($C_{16}H_{22}ClN_5O_4$) C, H, N.

6-Chloro-9*H*-(2-*C*-methyl-2,3-*O*-isopropylidene-β-D-ribofuranosyl)purine (14). A mixture of **4** (370 mg, 1.23 mmol), triethyl orthoformate (0.82 mL, 4.92 mmol), and *p*-toluenesulfonic acid monohydrate (220 mg, 1.16 mmol) in anhydrous acetone (12 mL) was stirred at room temperature for 3 h under nitrogen atmosphere. Evaporation of the reaction mixture gave a foam which was purified by flash chromatography on silica gel eluting with $CHCl_3$. Compound **14** was yielded as a white solid (69%): mp 115−118 °C. TLC ($CHCl_3$): R_f = 0.15. ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 1.6 (s, 3H, CH_3), 3.76 (t, J = 4.5 Hz, 2H, H-5′), 4.3 (m, 1H, H-4′), 4.65 (s, 1H, H-3′), 6.38 (s, 1H, H-1′), 8.83 (s, 1H, H-2), 8.95 (s, 1H, H-8). Anal. ($C_{14}H_{17}ClN_4O_4$) C, H, N.

1′-Deoxy-1′-(6-chloro-9*H*-purin-9-yl)-2′-*C*-methyl-2′,3′-*O*-isopropyl-idene-β-D-ribofuronic Acid (15). To a stirred solution of **14** (280 mg, 0.82 mmol) in acetone (5 mL) was added a solution of chromium trioxide (2 M) in aqueous H_2SO_4 (3 M, 0.8 mL) dropwise. After 2 h at room temperature, the dark mixture was filtered through Celite washing with EtOAc (2 × 10 mL). The filtrates were washed with 0.5 M $Na_2S_2O_5$ (5 mL) and water (3 × 5 mL) and then extracted with 0.5 M NaOH solution (10 mL). The extract was washed with EtOAc (10 mL) and acidified with concentrated HCl. Evaporation of the acidic solution gave a white solid residue which was washed with MeOH repeatedly and then filtered. The filtrate was evaporated in vacuo, and the residue was chromatographed on a silica gel column ($CHCl_3$−MeOH, 60:40) to give **15** as a white solid (90%): mp >240 °C dec. TLC ($CHCl_3$−MeOH, 80:20): R_f = 0.26. ^{1}H NMR (DMSO-d_6): δ 0.98 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 1.6 (s, 3H, CH_3), 4.5 (s, 1H, H-3′), 4.67 (s, 1H, H-4′), 6.45 (s, 1H, H-1′), 8.8 (s, 1H, H-2), 10.15 (s, 1H, H-8). Anal. ($C_{14}H_{15}ClN_4O_5$) C, H, N.

Ethyl-1′-Deoxy-1′-(6-chloro-9*H*-purin-9-yl)-2′-*C*-methyl-2′,3′-*O*-isopropylidene-β-D-ribofuranuroate (16). To a solution of **15** (250 mg, 0.7 mmol) in anhydrous EtOH (30 mL) at 0 °C was added dropwise 0.25 mL of $SOCl_2$, and the solution was stirred overnight under nitrogen atmosphere. After evaporation in vacuo, the residue was purified by chromatography on a silica gel column ($CHCl_3$−MeOH, 97:3). The appropriate fractions were collected and concentrated to yield **16** as a white solid (78%): mp 110−113 °C; TLC ($CHCl_3$−

MeOH, 97:3): $R_f = 0.16$. ^{1}H NMR (DMSO-d_6): δ 1.12 (t, J = 7.1 Hz, 3H, CH_3CH$_2$), 1.15 (s, 3H, CH$_3$), 1.45 (s, 3H, CH$_3$), 1.58 (s, 3H, CH$_3$), 4.6 (q, J = 7.1 Hz, 1H, CH$_3$CH_2), 4.97 (d, J = 2.1 Hz, 1H, H-3′), 5.25 (d, J = 2.3 Hz, 1H, H-4′), 6.52 (s, 1H, H-1′), 8.57 (s, 1H, H-2), 8.9 (s, 1H, H-8). Anal. ($C_{16}H_{19}ClN_4O_5$) C, H, N.

Ethyl 1′-deoxy-1′-[*N*^{6}-(3-iodobenzyl)-9*H*-purin-9-yl]-2′-*C*-methyl-2′,3′-*O*-isopropylidene-*β*-D-ribofuranuroate (17). To a stirred solution of **16** (190 mg, 0.5 mmol) in anhydrous CH$_3$CN (5 mL) were added 3-iodobenzylamine hydrochloride (200 mg, 0.75 mmol) and triethylamine (0.21 mL, 1.5 mmol) under nitrogen atmosphere. The reaction mixture was heated at 45 °C for 4 days. The solvent was evaporated to dryness, and the residue was purified by chromatography on a silica gel column eluting with CHCl$_3$ to give **17** as a foam (31%). TLC (CHCl$_3$−MeOH, 99:1): $R_f = 0.37$. ^{1}H NMR (DMSO-d_6): δ 1.03 (t, J = 7.0 Hz, 3H, CH_3CH$_2$), 1.18 (s, 3H, CH$_3$), 1.45 (s, 3H, CH$_3$), 1.55 (s, 3H, CH$_3$), 4.0 (m, 1H, CH$_3$CH_2), 4.75 (br s, 2H, N^6-CH$_2$Ph), 4.85 (d, J = 2.2 Hz, 1H, H-3′), 5.25 (d, J = 2.3 Hz, 1H, H-4′), 6.38 (s, 1H, H-1′), 7.12 (t, J = 7.7 Hz, 1H, Ph), 7.37 (d, J = 7.8 Hz, 1H, Ph), 7.6 (d, J = 7.9 Hz, 1H, Ph), 7.75 (s, 1H, Ph), 8.17 (s, 1H, H-2), 8.4 (s, 1H, H-8), 8.55 (pseudo t, 1H, NH). Anal. ($C_{23}H_{26}IN_5O_5$) C, H, N.

***N*-Methyl-1′-deoxy-1′-[*N*^{6}-(3-iodobenzyl)-9*H*-purin-9-yl]-2′-*C*-methyl-*β*-D-ribofuranuronamide (18).** A mixture of **17** (160 mg, 0.28 mmol) and anhydrous methylamine (2.54 mL) was stirred at −20 °C for 3 h and then at room temperature overnight. The solution was evaporated to dryness and the residue was used without further purification. TLC (CHCl$_3$−MeOH, 99:1): $R_f = 0.25$.

To the crude compound was added 1 N HCl (20 mL), and the resulting solution was heated at 55 °C for 3 h. Thereafter the mixture was allowed to cool with an ice bath, neutralized with aa aqueous NaHCO$_3$ solution, and extracted three times with ethyl acetate. The organic layer was dried in vacuo, and the residue was chromatographed on a silica gel column (CHCl$_3$−MeOH, 95:5) to give **18** as a white solid (69%): mp 230−233 °C dec TLC (CHCl$_3$−MeOH, 95:5): $R_f = 0.29$. ^{1}H NMR (DMSO-d_6): δ 0.8 (s, 3H, CH$_3$), 2.71 (d, J = 4.4 Hz, 1H, CH_3NH), 4.18 (pseudo t, 1H, H-3′), 4.28 (d, J = 7.7 Hz, 1H, H-4′), 4.68 (br s, NHCH_2Ph), 5.48 (s, 1H, OH), 5.64 (d, J = 6.2 Hz, 1H, OH), 6.0 (s, 1H, H-1′), 7.12 (t, J = 7.7 Hz, 1H, Ph), 7.37 (d, J = 7.4, 1H, Ph), 7.60 (d, J = 7.9 Hz, 1H, Ph), 7.75 (s, 1H, Ph), 8.25 (s, 1H, H-2), 8.55 (m, 2H, NH), 8.78 (s, 1H, H-8). Anal. ($C_{19}H_{21}IN_6O_4$) C, H, N.

9*H*-(2-*C*-Methyl-2,3-*O*-isopropylidene-*β*-D-ribofuranosyl)adenine (19). A mixture of **5** (380 mg, 1.35 mmol), 2,2-dimethoxypropane (3.0 mL, 24.5 mmol), *p*-toluenesulfonic acid (250 mg, 1.35 mmol), and acetone (12 mL) was stirred for 16 h at room temperature. The solvent was removed in vacuo, and the residue was chromatographed on a silica gel column (CHCl$_3$−MeOH, 92:8). The appropriate fractions were collected and concentrated to give **19** as a white solid (30%): mp >240 °C. TLC (CHCl$_3$−MeOH, 97:3): $R_f = 0.18$. ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 3H, CH$_3$), 1.38 (s, 3H, CH$_3$), 1.6 (s, 3H, CH$_3$), 3.73 (q, J = 5.1 Hz, 2H, H-5′), 4.25 (m, 1H, H-4′), 4.6 (s, 1H, H-3′), 5.42 (t, J = 5.5 Hz, 1H, OH), 6.2 (s, 1H, H-1′), 7.3 (br s, 2H, NH$_2$), 8.17 (s, 1H, H-2), 8.35 (s, 1H, H-8). Anal. ($C_{14}H_{19}N_5O_4$) C, H, N.

1′-Deoxy-1′-(6-amino-9*H*-purin-9-yl)-2′-*C*-methyl-2′,3′-*O*-isopropylidene-*β*-D-ribofuronic Acid (20). A mixture of **19** (120 mg, 0.38 mmol), aqueous solution of KOH (64 mg, 1.14 mmol in 4 mL), and aqueous solution of KMnO$_4$ (180 mg, 1.14 mmol in 8.7 mL) was vigorously stirred for 8 h at room temperature. The reaction was quenched by addition of 7.5% aqueous H$_2$O$_2$ solution (4.4 mL), and then the mixture was filtered through Celite. After concentration in vacuo the residue was acidified to pH 4 with 3 N HCl. The precipitate obtained was collected and dried to give **20** as a chromatographically pure white solid (79%): mp >240 °C. TLC (CHCl$_3$−MeOH, 60:40): $R_f = 0.35$. ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 3H, CH$_3$), 1.42 (s, 3H, CH$_3$), 1.6 (s, 3H, CH$_3$), 4.8 (s, 1H, H-3′), 5.08 (s, 1H, H-4′), 6.35 (s, 1H, H-1′), 7.4 (br s, 2H, NH$_2$), 8.12 (s, 1H, H-2), 8.45 (s, 1H, H-8), 13.6 (br s, 1H, COOH). Anal. ($C_{14}H_{17}N_5O_5$) C, H, N.

Ethyl 1′-Deoxy-1′-(6-amino-9*H*-purin-9-yl)-2′-*C*-methyl-2′,3′-*O*-isopropylidene-*β*-D-ribofuranuroate (21). The title compound was synthesized from **20** (180 mg, 0.54 mmol) as described for **16**. The residue was purified by chromatography on a silica gel column (CHCl$_3$−MeOH, 95:5) to yield a white solid (60%): mp 180−182 °C. TLC (CHCl$_3$−MeOH, 95:5): R_f = 0.5. ^{1}H NMR (DMSO-d_6): δ 1.12 (t, J = 7.1 Hz, 3H, CH_3-CH$_2$), 1.17 (s, 3H, CH$_3$), 1.45 (s, 3H, CH$_3$), 1.55 (s, 3H, CH$_3$), 4.0 (m, 2H, CH$_3$CH_2), 4.85 (d, J = 2.4 Hz, 1H, H-3′), 5.30 (d, J = 2.5 Hz, 1H, H-4′), 6.46 (s, 1H, H-1′), 7.38 (br s, 2H, NH$_2$), 8.08 (s, 1H, H-2), 8.34 (s, 1H, H-8). Anal. ($C_{16}H_{21}N_5O_5$) C, H, N.

***N*-Ethyl-1′-deoxy-1′-[*N*^{6}-(3-iodobenzyl)-9*H*-purin-9-yl]-2′-*C*-methyl-*β*-D-ribofuranuronamide (22).** The title compound was obtained from **21** (100 mg, 0.28 mmol) by the same methodology described for **18**. The crude compound was purified by chromatography on a silica gel column (CHCl$_3$−MeOH, 90:10) to give **22** as a white solid (90%): mp 192−195 °C. TLC (CHCl$_3$-MeOH, 90:10): $R_f = 0.35$. ^{1}H NMR (DMSO-d_6): δ 0.8 (s, 3H, CH$_3$), 1.1 (t, J = 7.3 Hz, 3H, CH_3CH$_2$), 3.2 (m, 2H, CH_3CH$_2$), 4.18 (pseudo t, 1H, H-3′), 4.28 (d, J = 7.7 Hz, 1H, H-4′), 5.48 (s, 1H, OH), 5.64 (d, J = 6.2 Hz, 1H, OH), 6.0 (s, 1H, H-1′), 7.40 (br s, 2H, NH$_2$), 8.18 (s, 1H, H-2), 8.7 (s, t, 2H, NH, H-8). Anal. ($C_{13}H_{18}N_6O_4$) C, H, N.

Biological Methods. 1. Materials. [^{3}H]-(*R*)-PIA, [^{3}H]-cAMP (25 Ci/mmol), and [α-^{32}P]ATP (30−40 Ci/mmol) were from Amersham Corp. (Little Chalfont, Buckinghamshire, U.K.), while [^{3}H]CHA and [^{3}H]CGS 21680 were obtained from DuPont-NEN (Boston, MA). CHA, CPA, and DPCPX were purchased from RBI (Natik, MA). Adenosine deaminase, forskolin, and GTP were from Sigma Chemical Co. (St. Louis, MO). Myokinase and creatine kinase were purchased from Boehringer-Mannheim (Mannheim, Germany). All other reagents were from standard commercial sources and of the highest grade commercially available.

2. Receptor Binding Assay. Bovine brains were obtained from the local slaughterhouse. Cortex and striatum were dissected from bovine brain and immediately homogenized. Cerebral cortex was homogenized in 10 volumes of ice-cold buffer containing 0.25 M sucrose, 5 mM EDTA, 0.1 mM PMSF, 200 mg/mL bacitracin, 160 μg/mL benzamidine, and 10 mM TRIS/HCl, pH 7.7. The membrane homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 46000*g* for 20 min at 4 °C. The pellet was resuspended in 10 volumes of ice-cold buffer A (1 mM EDTA, 5 mM MgCl$_2$, and 50 mM TRIS/HCl, pH 7.7) containing protease inhibitors (as above) and centrifuged at 46000*g* for 20 min at 4 °C. The pellet was resuspended in 5 volumes of buffer A containing protease inhibitors (as above) and adenosine deaminase (2 units/mL). After incubation for 30 min at 37 °C, the suspension was centrifuged at 46000*g* for 20 min at 4 °C. The final pellet was stored in aliquots at −80 °C until the time of assay. Cortical membranes were resuspended in buffer A, and binding of [^{3}H]CHA (31 Ci/mmol) to A$_1$ receptors was measured in triplicate as previously described.[12a]

Striatal tissue was homogenized in 20 volumes of ice-cold 1 mM EDTA, 10 mM MgCl$_2$, 50 mM TRIS/HCl buffer, pH 7.4 (buffer B), containing protease inhibitors (as above). The homogenate was centrifuged at 46000*g* for 10 min at 4 °C. The resulting pellet was resuspended in 20 volumes of buffer B containing protease inhibitors (as above) and adenosine deaminase (2 units/mL). Incubation was carried out for 30 min at 37 °C. The membrane suspension was recentrifuged, and the final pellet was frozen in aliquots at −80 °C until the time of assay. Striatal membranes were resuspended in buffer B, and binding of [^{3}H]CGS 21680 (41.2 Ci/mmol) to A$_{2A}$ receptors was performed in triplicate as previously described.[12b]

A$_3$ receptor binding sites were studied in rat testis membranes. Fresh testicular tissue from Sprague−Dawley rats was dissected free of epididymis and membranes were prepared as described.[13] Binding of [^{3}H]-(*R*)-PIA (38 Ci/mmol) to membranes was measured in the presence of DPCPX (150 nM) as previously described.[13] Briefly, rat testis membranes (100−200 μg of protein) and [^{3}H]-(*R*)-PIA (2 nM) were incubated in 0.5 mL total volume of 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 10 mM MgCl$_2$ buffer in the presence of 150 nM DPCPX to block A$_1$ adenosine receptors. Nonspecific binding was determined in the presence of 15 μM (*R*)-PIA.

Compounds were routinely dissolved in DMSO and then diluted with assay buffer, and final DMSO concentrations never exceeded 1%. At least six different concentrations spanning 3 orders of magnitude, adjusted appropriately for the IC_{50} of each compound, were used. IC_{50} values, computer-generated using a nonlinear regression formula on a computer program (GraphPad, San Diego, CA), were converted to K_i values, knowing the K_d values of radioligands in these different tissues and using the Cheng and Prusoff equation.[20]

3. Adenylyl Cyclase Assay. Cerebral cortex was obtained from male Sprague−Dawley rats sacrificed by cervical dislocation. Fresh tissue was suspended in 50 volumes of ice-cold buffer containing 0.32 M sucrose, 200 μg/mL bacitracin, 160 μg/mL benzamidine, 20 μg/mL trypsin inhibitor, 0.1 mM PMSF, and 10 mM HEPES/NaOH, pH 7.4, and homogenized with 12 strokes of a Teflon homogenizer at 4 °C. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was collected and centrifuged at 48000*g* for 30 min at 4 °C. The pellet was resuspended in 10 volumes of an ice-cold hypotonic buffer containing all the constituents of the homogenization buffer except sucrose and incubated with adenosine deaminase (2 units/mL) for 30 min at 30 °C. The membrane suspension was centrifuged at 48000*g* for 15 min at 4 °C. This centrifugation step was repeated twice. The final pellet was resuspended in 50 mM HEPES/NaOH, pH 7.4, and used in adenylyl cyclase assays.

Adenylyl cyclase activity was measured by monitoring the conversion of [α-^{32}P]ATP to [α-^{32}P]cAMP, using a previously reported method.[21] The method involved addition of [α-^{32}P]-ATP to membranes in the presence of forskolin to stimulate adenylyl cyclase and papaverine as a phosphodiesterase inhibitor. Briefly, enzyme activity was routinely assayed in a 100-μL reaction mixture containing 50 mM HEPES/NaOH buffer, pH 7.4, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL creatine phosphokinase, 0.1 mg/mL bacitracin, 0.5 mg/mL creatine phosphate, 0.1 mM ATP, 0.05 mM cAMP, 15 units/mL myokinase, 2 units/mL adenosine deaminase, 10 μM GTP, 1 μCi [α-^{32}P]ATP, 0.2 mM papaverine, and 0.1 mM forskolin. The incubation was started by the addition of membranes (10−20 mg of protein) and carried out for 15 min at 23 °C. The reaction was terminated by placing assay tubes into an ice bath and adding 0.5 mL of a stop solution containing 120 mM $Zn(C_2H_3O_2)_2$/[^{3}H]cAMP (10 000−20 000 cpm/sample) and then 0.5 mL of 144 mM Na_2CO_3. The total radiolabeled cAMP was isolated on columns of Dowex 50 ion-exchange resin and alumina as described.[21]

Compounds tested as inhibitors of forskolin-stimulated adenylyl cyclase activity were dissolved in DMSO and then diluted with 50 mM HEPES/NaOH buffer, pH 7.4, so the final DMSO concentration never exceeded 1%. Maximal inhibition of adenylyl cyclase activity corresponded to 10−20% of total activity under conditions of stimulation (typically by 3−4-fold) in the presence of 0.1 mM forskolin. IC_{50} values were calculated using a nonlinear regression analysis (GraphPad).

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