macromolecules. Attached V79 cells were cultured in 60-mm plastic culture dishes, to a density of about 1×10^5 cells/cm^2 at which time the cells were undergoing a logarithmic rate of growth. Growth medium was then replaced with fresh medium containing [^{3}H]TdR ([*methyl*,1′,2′-^{3}H]thymidine: 95 Ci/mmol, 2 μCi/mL, New England Nuclear, Boston, MA), and incubation at 37 °C was resumed for 10 min. DNA synthesis inhibitors were then added from freshly prepared stock solutions: hydroxyurea was dissolved in balanced salt solution, and NBT or its iron chelate was dissolved and diluted in dimethyl sulfoxide (DMSO). Control samples included untreated cells and cells treated with appropriate concentrations of the vehicle alone.

After incubation in the presence of chelators for 0–120 min, the dishes were removed from the incubator and placed on ice, and ice-cold 70% percholoric acid (PCA) was added to a final concentration of 0.5 N in the previously incubated balanced salt solution. After 60 min, the precipitated cellular macromolecules were transferred to a Gelman type A/E glass fiber filter, which was washed twice with 5 mL of cold PCA. The radioactivity on the filters was then counted and converted to ^{3}H dpm using a Beckman LS-9800 liquid scintillation spectrometer.

Alkaline Elution of DNA. DNA damage was measured by the alkaline elution technique,[23] as modified by Moss et al. and described elsewhere.[18,24,25] Briefly, control V79 lung fibroblast cells labeled with [^{3}H]TdR and experimental cells labeled with [^{14}C]TdR (New England Nuclear, Boston, MA) each were added to a lysing solution in a filter assembly containing cellulose triacetate filter membranes. The two DNA populations were then coeluted with tetrapropylammonium hydroxide. The filter eluate was collected with 1-mL fractions, and the radioactivity per fraction was measured and expressed as ^{3}H and ^{14}C dpm. Under these conditions, about 85% of the DNA from untreated cells eluted from the filters; thus, there was no need to use irradiated cells as a source of reference DNA for coelution.

DNA damage was measured as the mass of DNA from treated cells (proportional to the ^{14}C dpm) eluting prior to the DNA from untreated control cells (proportional to the ^{3}H dpm). The damage is expressed as the "elution parameter", which is directly proportional to the number of DNA lesions expressed as strand breaks in alkali, i.e., DNA strand scissions plus alkali-labile sites.[24]

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Registry No. 1, 51618-01-0; 2, 49608-51-7; 3, 119437-72-8; 4, 78217-68-2; 5, 119437-73-9; 6, 119437-74-0; 7, 119437-75-1; *N*-hydroxysuccinimide, 6066-82-6.

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Electrophilic Derivatives of Purines as Irreversible Inhibitors of A_1 Adenosine Receptors

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Functionalized congeners derived from 1,3-dipropyl-8-phenylxanthine and from N^6-phenyladenosine were derivatized to contain electrophilic groups (isothiocyanate, *N*-hydroxysuccinimide ester, maleimide, sulfonyl chloride, or α-haloacyl group) capable of reaction with nucleophiles on biopolymers. The goal was to inhibit chemically the A_1 adenosine receptor by using reactive agonist and antagonist ligands. Some of the electrophilic derivatives were synthesized through acylation of amine-functionalized congeners using hetero- or homobifunctional reagents available for protein cross-linking. The affinity for A_1 adenosine receptors was evaluated in competitive binding assays by using rat and bovine brain membranes. Several xanthine and adenosine thiourea derivatives prepared from 1,3- and 1,4-phenylene diisothiocyanate (DITC) were potent irreversible inhibitors of adenosine receptors. Derivatives of *m*-DITC, at concentrations between 10 and 500 nM, irreversibly eliminated binding at 90% of the A_1-receptor sites. Receptor affinity of both xanthine and adenosine derivatives containing distal phenylthiourea substituents was diminished by electron-donating groups on the ring.

Adenosine modulates a variety of physiological functions.[1] It acts as an inhibitor of neuronal firing and the release of neurotransmitters,[1b] an inhibitor of platelet aggregation,[1c] a cardiac depressant and a vasodilator,[1d] a vasoconstrictor[1e] (e.g., in the renal afferent arterioles and in the skin), and an immunosuppressant.[1f] Most of the physiological effects of adenosine result from binding to discrete membrane-bound adenosine receptors of the A_1 or A_2 subtypes.[1g] The xanthine drugs caffeine and theophylline, and many synthetic analogues,[2] act as competitive antagonists at adenosine receptors.

Alkylating or acylating ligands that form a stable covalent bond with a receptor have been synthesized for a number of receptors, including opiate,[3,4] phencyclidine,[5]

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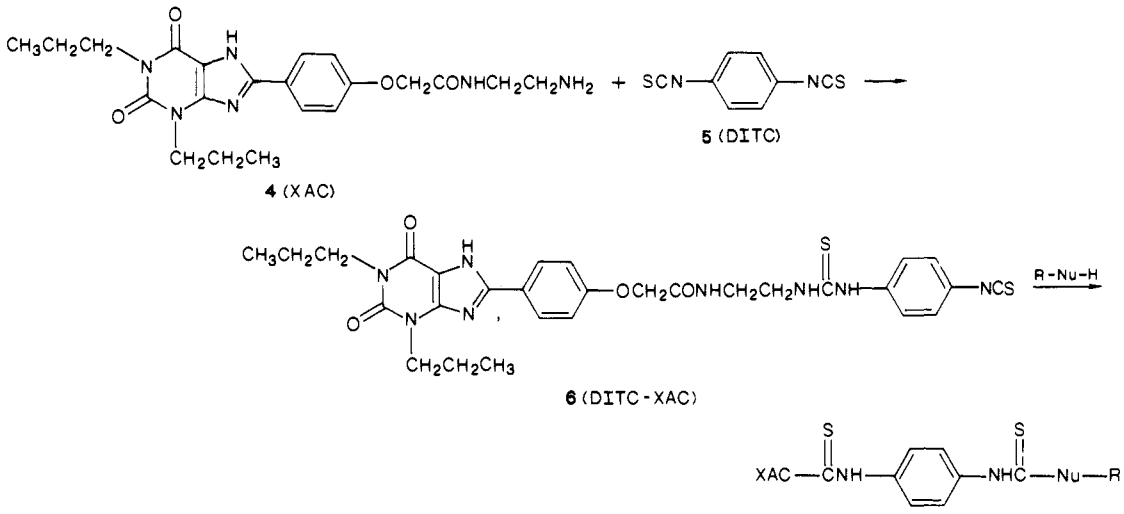
0022-2623/89/1832-1043$01.50/0

Scheme I



7, R-Nu-H = a nucleophilic amine (RNH_2) or thiol (RSH) group on a biopolymer
8, R-Nu-H = $H_2N(CH_2)_2NH_2$

adrenergic,[6] and benzodiazepine receptors.[7] The utility of such chemically irreversibly bound ligands for characterizing receptors in membranes and in physiological systems,[4,5] and in receptor identification,[8] has been demonstrated. For adenosine receptors, only photoaffinity labels[1g,9] have hitherto been described. We now report a set of reactive adenosine receptor ligands, derived from agonist[10] and antagonist[11] functionalized congeners. These ligands contain electrophilic acylating and alkylating groups capable of reaction with nucleophilic residues in proximity to the adenosine receptor binding site.

Results

Synthesis. We have synthesized a variety of high-affinity ligands, based on 8-aryl-substituted xanthines (Table I) and on N^6-substituted adenosine (Table II), potentially capable of irreversible reaction with adenosine receptors. The ligands contain electrophilic groups such as (1) isothiocyanate (**6**, **10**, **26**, **29**, **44–46**) or bromoacetyl (**31** and **56**), both of which are reactive with either amines or thiols; (2) pyridyl disulfide (**37**) and maleimide (**38** and **39**), reactive with thiols; or (3) *N*-hydroxysuccinimide esters (**1**, **34–36**, **40**, **41**), reactive with amines. Aldehyde (**3**) and ketone (**30**) derivatives are potentially reactive with amines via the formation of Schiff bases.

The compounds are related to previously reported purine "functionalized congeners". These chain-derivatized drug analogues act as adenosine agonists (N^6-phenyladenosine derivatives) or antagonists (1,3-dipropyl-8-phenylxanthine derivatives).[10-12] 8-[4-[[[[(2-Aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (**4**; XAC) has been characterized as A_1 selective in reversing cardiac depressant effects of adenosine agonists[1d] and in renin release[13] and as a radioligand having nanomolar affinity.[11] N^6-[4-[[[4-[[[(2-Aminoethyl)amino]carbonyl]methyl]anilino]carbonyl]methyl]phenyl]adenosine (**43**; ADAC) has also been characterized as a high-affinity ligand for adenosine receptors and synthetic intermediate for molecular probes.[10]

Some of the compounds (**6**, **31**, **35–38**, **45**, and **56**) were synthesized from the amine congeners, compounds **4** and **43**, with commercially available hetero- and homobifunctional cross-linking reagents (Table III). For example, a phenylthiourea derivative bearing an isothiocyanate group, compound **6** (*p*-DITC-XAC), was prepared (Scheme I) through acylation of compound **4**[11] in the presence of a large molar excess of the homobifunctional cross-linking reagent 1,4-phenylene diisothiocyanate (**5**; DITC). The isothiocyanate group is intended for reaction with a hypothetical nucleophilic residue, i.e., an amine or thiol, of the A_1 adenosine receptor to form a thiourea or dithiourethane, respectively (**7**). Compound **5** has been used previously for (1) cross-linking of the myosin subfragment 1,[14] (2) cross-linking proteins and phosphatidylethanolamine in membranes,[15] and (3) attaching peptides to resins

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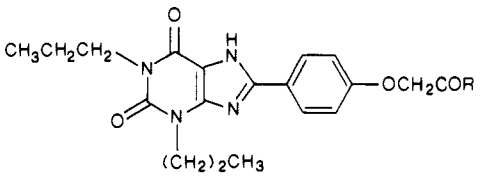
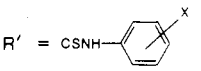
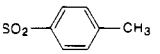
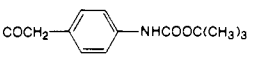
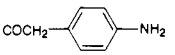
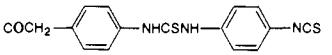
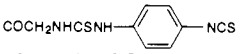
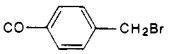
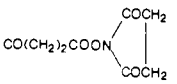
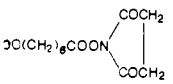
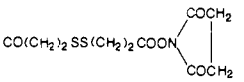
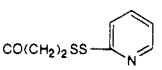
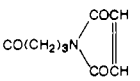
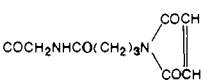
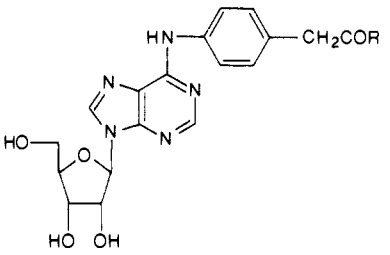
Table I. Xanthine Derivatives

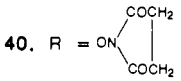
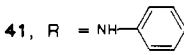
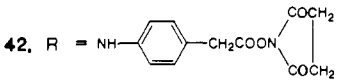
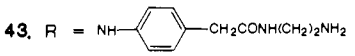
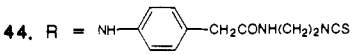
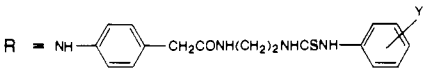
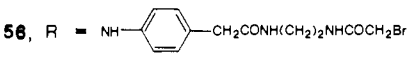


compd no. and structure	K_i at central A_1 receptors, nM: rat[a]	calf[b]	% inhibn (concn)[c]
1, R = 	9.0 ± 0.7[d]	*e*	10 (50)
3, R = $NHCH_2CHO$	266 ± 40[d]	3.17 ± 0.4	0 (500)
	R = $NH(CH_2)_2NHR'$		
4, R′ = H	1.1 ± 0.05	0.3 ± 0.05	*e*
	R′ = 		
6, X = 4-NCS	6.60 ± 1.31	1.3 ± 0.2	29 (50) 63 (100) 73 (500)
10, X = 3-NCS	2.39 ± 0.35	*e*	49 (5) 67 (50) 94 (500)
11, X = 4-CH_3	16.0 ± 2.9	*e*	*e*
12, X = 4-OCH_3	33.2 ± 3.47	*e*	*e*
13, X = 4-F	15.5 ± 1.68	*e*	*e*
14, X = 2,3,4,5,6-F_5	9.07 ± 0.90	*e*	*e*
15, X = 4-CN	16.6 ± 1.69	*e*	*e*
16, X = 4-Br	7.26 ± 0.90	*e*	*e*
17, X = 4-NO_2	15.9 ± 2.36	*e*	*e*
18, X = 4-SO_3Na	367 ± 73.4	*e*	*e*
19, R′ = 	2.5 ± 0.47	*e*	23 (50)
20, R′ = 	72 ± 7.2[d]	*e*	*e*
24, R′ = 	53 ± 12	*e*	*e*
25, R′ = 	40	*e*	*e*
26 R′ = 	25.0 ± 6.6	*e*	20 (50)
29, R′ = 	6.85 ± 1.14	*e*	50 (50)
30, R′ = $CO(CH_2)_4COCH_3$	44 ± 9	6.2 ± 0.7	0 (500)
31, R′ = $COCH_2Br$	17.6 ± 1.5[d]	*e*	16 (50)
32, R′ = 	11.5 ± 1.5[d]	10.1 ± 1.2	15 (50)
34, R′ = 	20.0 ± 4.5	*e*	0 (500)
35, R′ = 	3.69 ± 0.71	23.0 ± 2.0	32 (50)
36, R′ = 	54.9 ± 8.6	*e*	0 (50)
37, R′ = 	23.8 ± 3.51	*e*	3 (50)
38 R′ = 	37.8 ± 4.38	*e*	15 (50)
39, R′ = 	23.2 ± 2.14	*e*	9 (50)

[a] Against [^{3}H]PIA, ±SEM; for compounds that inhibit irreversibly, the value represents an apparent K_i, since a fraction of the sites are inactivated. [b] Against ^{125}I-APNEA, ±SEM. [c] Percent inhibition following a 45-min incubation with the compound at the concentration given in parentheses (nM) and washout, in rat brain membranes, against ^{125}I-APNEA. [d] Values taken from references 11b and 12. [e] Not determined.

Table II. Adenosine Derivatives



compd no. and structure	K_i at central A_1 receptors, nM		
	rat[a]	calf[b]	% inhibn (concn)[c]
40, R = 	8.37 ± 0.73	*d*	21 (50)
41, R = 	*d*	*d*	*d*
42, R = 	9.60 ± 1.44	*d*	44 (50)
43, R = 	1.3 ± 0.3	*d*	*d*
44, R = 	4.5	4.2 ± 0.5	*d*
R = 			
45, Y = 4-NCS	0.469 ± 0.13	*d*	39 (1) >80 (10)
46, Y = 3-NCS	0.867 ± 0.114	*d*	62 (1) >90 (10)
47, Y = 4-CH_3	3.72 ± 0.44	*d*	*d*
48, Y = 4-OCH_3	8.52 ± 0.85	*d*	*d*
49, Y = 4-F	8.71 ± 1.66	*d*	*d*
50, Y = 2-F	6.75 ± 1.97	*d*	*d*
51, Y = 2,3,4,5,6-F_5	1.64 ± 0.47	*d*	*d*
52, Y = 4-CN	2.66 ± 0.22	*d*	*d*
53, Y = 4-Br	1.19 ± 0.14	*d*	*d*
54, Y = 4-NO_2	1.97 ± 0.50	*d*	*d*
55, Y = 4-SO_3Na	51.1 ± 7.81	*d*	*d*
56, R = 	8.30 ± 0.45	*d*	38 (50)

[a] Against [^{3}H]PIA, ±SEM; for compounds that inhibit irreversibly, the value represents an apparent K_i, since a fraction of the sites are inactivated. [b] Against ^{125}I-APNEA, ±SEM. [c] Percent inhibition following a 45-min incubation with the compound at the concentration given in parentheses (nM) and washout, in rat brain membranes, against ^{125}I-APNEA. [d] Not determined.

for stepwise degradations.[16] Similarly, the meta isomer **10** was prepared from 1,3-phenylenediisothiocyanate (**9**). Thus, *m*- and *p*-isothiocyanate xanthine derivatives are related to a class of potent and moderately A_1 selective[1d,11,13] adenosine antagonists derived from compound **4**.

Two variations of compound **6**, containing additional spacer groups, e.g., **26** [(*p*-aminophenyl)acetyl] and **29** (glycyl), were prepared. In each case the final synthetic step (Scheme II) consisted of treatment of an amine-functionalized xanthine with excess 1,4-phenylene diisothiocyanate (**5**). A precursor of **26**, (*tert*-butyloxycarbonyl)-*p*-aminophenylacetic acid (**22**) was first converted to the *N*-hydroxysuccinimide ester **23**, which reacted with the amine **4**. The aryl amine **25** (PAPA-XAC) has been reported previously as a precursor for iodination in the preparation of a radioligand.[9] The synthetic route for compound **25** used here gave better results than the previously reported method,[9] which involved catalytic reduction of an aryl nitro group as the final step.

Sulfonyl halides are also reactive toward nucleophilic amino acid residues. Compound **19**, a derivative of benzene-1,3-disulfonyl chloride, monosubstituted with the amine of compound **4**, was prepared.

Several *N*-hydroxysuccinimide esters, e.g., **34** and **40**, have been prepared from purine carboxylic acid derivatives, compound **33** (the *N*-succinoyl derivative of compound **4**) and N^6-[(carboxymethyl)phenyl]adenosine,[10] respectively, by using a water-soluble carbodiimide and *N*-hydroxysuccinimide. In an alternate synthetic approach, compound **35** was prepared by treating the xanthine amine congener **4** with excess disuccinimidyl suberate (DSS), a protein cross-linker. An alkyl isothiocyanate derivative of adenosine, **44**, has been prepared by the reaction of thiophosgene with the amine **43**. Bromoacetyl derivatives, **31** and **56**, were prepared conveniently by the reaction of amine **4** or **43** with excess bromoacetic anhydride.

Compound **36**, derived from disuccinimidyl dithiobis(propionate), was designed as a thiol-cleavable ligand

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Table III. Analytical Data

no.	method[a]	% yield	mp, °C	formula	anal.
2	A	57	238–239	$C_{23}H_{31}H_5O_6$	C, H, N
3	B	76	203–206	$C_{21}H_{25}N_5O_5$	*d*
6	C	58	140[d]	$C_{29}H_{32}N_8O_4S_2 \cdot H_2O$	C, H, N
9	D	81	50.5–51	$C_8H_4N_2S_2$	C, H, N
10	C	62	186–188	$C_{29}H_{32}N_8O_4S.0.5H_2O$	C, H, N
11	C	87	205–208	$C_{29}H_{35}N_7O_4S$	C, H, N
12	C	89	170–174	$C_{29}H_{35}N_7O_5S \cdot H_2O$	C, H, N
13	C	89	163–165	$C_{28}H_{32}N_7O_4FS$	C; H, N[c]
14	C	58	257–265	$C_{28}H_{28}N_7O_4F_5 \cdot 0.5H_2O$	C, H, N
15	C	84	249–251	$C_{29}H_{32}N_8O_4S$	C, H, N
16	C	70	205–208	$C_{28}H_{32}N_7O_4BrS$	C, H, N
17	C	93	248–250	$C_{28}H_{32}N_8O_6S \cdot 0.5H_2O$	C, H, N
18[b]	C	64	275[d]	$C_{28}H_{32}N_7O_7S_2Na \cdot 2H_2O$	H; C, N[c]
19	E	57	256–260	$C_{27}H_{31}N_6O_8S_2Cl$	*e*
20	E	90	211–216	$C_{28}H_{34}N_6O_6S \cdot 0.5H_2O$	C, H, N
22	F	40	141.5–142.5	$C_{13}H_{17}NO_4$	C, H, N
23	A	69	169–170	$C_{17}H_{20}N_2O_6$	C, H, N
24	G	75	215–220	$C_{34}H_{43}N_7O_7 \cdot 0.5H_2O$	C, H, N
25	B	89	204–208	$C_{29}H_{35}N_7O_5 \cdot 2H_2O$	C, H, N
26[b]	C	50	194–204	$C_{37}H_{39}N_9O_5S_2 \cdot H_2O$	C, H, N
27	A	81	210–212	$C_{28}H_{39}N_7O_7 \cdot H_2O$	C, H, N
28	B	100	200–210	$C_{25}H_{32}N_7O_7F_3 \cdot DMF$	H, N; C[c]
29	C	90	173–175	$C_{31}H_{35}N_9O_5S_2 \cdot H_2O$	C, H; N[c]
30	A	33	236–239	$C_{28}H_{38}N_6O_6$	C, H, N
31	F	93	275–279	$C_{23}H_{29}N_6O_5Br \cdot H_2O$	C, H, N
32[b]	G	78	277–280	$C_{29}H_{33}N_6O_5Br$	*f*
33	F	80	210–212	$C_{25}H_{32}N_6O_7 \cdot 1.5H_2O$	C, H, N
34	A	83	161–162	$C_{29}H_{35}N_7O_9 \cdot H_2O$	C, H, N
35	G	92	189–197	$C_{33}H_{43}N_7O_9 \cdot DMF$	C, H, N
36	G	46	219–224	$C_{31}H_{39}N_7O_9S_2 \cdot 2DMF$	H, N; C[c]
37	G	83	212–219	$C_{29}H_{35}N_7O_5S_2$	C, H, N
38	G	74	203–206	$C_{29}H_{35}N_7O_7 \cdot 0.5H_2O$	C, H, N
39	G	70	192–195	$C_{31}H_{38}N_8O_8 \cdot 0.5H_2O$	C, H, N
40	A	41	132–135	$C_{22}H_{22}N_6O_8 \cdot 1.5H_2O$	C, H, N
41	H	86	182–184	$C_{26}H_{26}N_6O_7 \cdot 1.5H_2O$	C, H, N
42	A	74	175[d]	$C_{30}H_{29}N_7O_9$	*d*
44[b]	D	48		$C_{29}H_{30}N_8O_6S$	*d*
45	C	77	179–181	$C_{36}H_{36}N_{10}O_6S_2 \cdot H_2O$	C, H, N
46	C	78	182–183	$C_{36}H_{36}N_{10}O_6S_2$	C, H, N
47	C	90	178–182	$C_{36}H_{39}N_9O_6S \cdot 0.5H_2O$	C, H, N
48	C	95	171–174	$C_{36}H_{39}N_9O_7S \cdot H_2O$	C, H, N
49	C	91	171–174	$C_{35}H_{36}N_9O_6FS \cdot 2H_2O$	C, H, N
50	C	63	169–174	$C_{35}H_{36}N_9O_6FS \cdot 2H_2O \cdot 2DMF$	C, H, N
51[b]	C	40		$C_{35}H_{32}N_9O_6F_5S \cdot 0.5H_2O$	C, H, N
52	C	96	171–173	$C_{36}H_{36}N_{10}O_6S$	C, H, N
53	C	87	178–182	$C_{35}H_{36}N_9O_6BrS$	C, H, N
54	C	99	172–175	$C_{35}H_{36}N_{10}O_8S \cdot 0.5H_2O$	C, H, N
55[b]	C	51	219–229	$C_{35}H_{36}N_9O_9S_2Na \cdot H_2O$	C, H, N
56[b]	F	91	dec 220	$C_{30}H_{33}N_8O_7Br \cdot H_2O$	C, H, N
57	D	93	118–120	$C_8H_4N_2S$	C, H, N

[a] Key to synthetic methods: (A) carbodiimide coupling; (B) acid cleavage; (C) thiourea from amine and isothiocyanate; (D) isothiocyanate from amine and thiophosgene; (E) sulfonamide from amine and sulfonyl chloride; (F) acylation of amine; (G) amide from amine and *N*-hydroxysuccinimide ester; (H) ester saponification. [b] Identified by californium plasma desorption mass spectroscopy,[23] intense M + Na^+ ion observed for compounds **26**, **32**, **44**, **51**, and **56**. Peaks corresponding to loss of H_2S observed for compounds 18 and **55**. [c] For compound 13: (H) calcd, 5.55; found, 5.04; (N) calcd, 16.86; found, 14.46. For compound 18: (C) calcd, 50.52; found, 44.45; (N) calcd, 14.73; found, 12.59. For compound 18: (C) calcd, 50.00; found, 50.84. For compound **29**: (N) calcd, 18.12; found, 17.49. For compound **36**: (C) calcd, 51.44; found, 52.88. [d] Characterization under Materials and Methods. [e] Characteristic NMR resonances at 8.30 (t, 1 H, NH), 7.90 (t, 1 H, C_2Ar, J = 1.6 Hz), 7.72 (br, 2 H), 7.52 (d, 1 H, J = 7.7 Hz, ortho to S), 7.26 (t, 1 H, J = 7.4 Hz, meta to S) ppm. [f] Characteristic NMR resonances at 7.80 (8.3 Hz), 7.48 (8.0 Hz), 4.77 (s, 2 H, CH_2Ar) ppm.

analogue of **35**. Any degree of irreversible inhibition of the receptor through incorporation of **36** would be reversible in a subsequent reductive chemical step to cleave the disulfide linkage.

The reaction of compound **4** with the homobifunctional reagents disuccinimidyl tartrate and *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester yielded predominantly side products, and the desired conjugates were not isolated.

Biology. The xanthine and adenosine derivatives were assayed for affinity at A_1 adenosine receptors in rat brain membranes (Tables I and II), by use of [^{3}H]-N^6-(phenylisopropyl)adenosine [(*R*)-[^{3}H]PIA] as a radioligand, and were found to have high affinity for this class of receptors. The affinity for A_1 adenosine receptors in bovine brain was also determined in competitive binding assays. As observed previously,[17] many derivatives of N^6-phenyladenosine and of 1,3-dialkyl-8-phenylxanthine were more potent in bovine brain than in rat brain.

The most potent of the potentially irreversibly binding xanthines were **10** ≈ **19** > **35** > **6** ≈ **29**. Compound **19** was 29-fold more potent than the closely related *p*-toluenesulfonamide **20**. The suberoyl active ester **35** was 5.4-fold more potent than the smaller, corresponding succinoyl derivative **34**.

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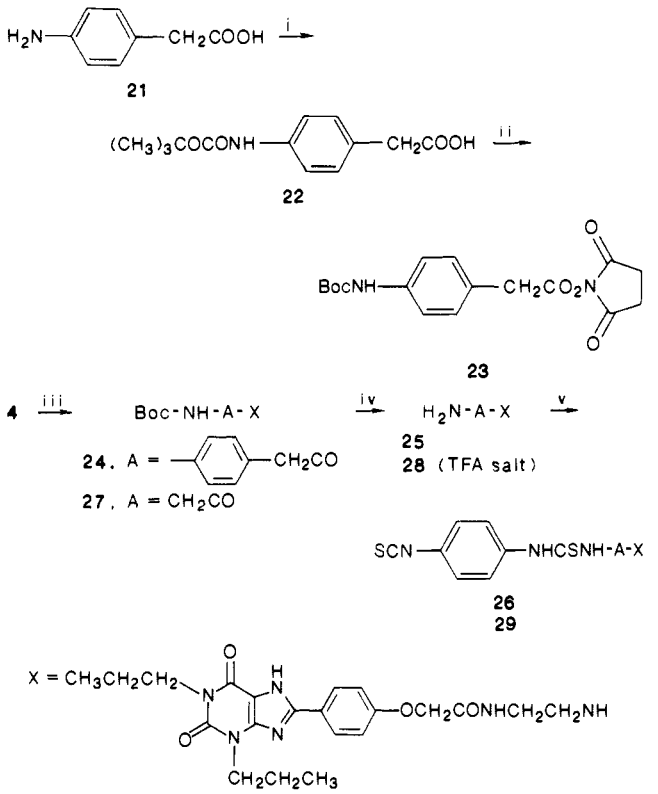
Scheme II[a]



[a] Reagents: (i) di-*tert*-butyl dicarbonate; (ii) EDAC, *N*-hydroxysuccinimide; (iii) either Boc-Gly-OH/EDAC/HOBt (for **27**) or **23** (for **24**); (iv) trifluoroacetic acid (TFA); (v) 1,4-phenylene diisothiocyanate.

The most potent of the electrophilic adenosine derivatives was compound **45** (*p*-DITC-ADAC), which was greater than 2-fold more potent than the parent amine **43**. Compound **46** (*m*-DITC-ADAC) was equipotent with **43**. The *N*-hydroxysuccinimide esters **40** and **42** were of equal potency, in spite of the added bulk of the longer chain derivative.

In previous studies,[10,11] parallels in SAR between agonists and antagonists (the distal amine sites of compounds **4** and **43**) have been noted. Phenylenediisothiocyanate derivatives of compounds **4** and **43** were highly potent in competitive binding experiments relative to most other neutral, acylated analogues. To investigate the structure–activity relationships for thiourea derivatives, two series of ring-substituted phenylthiourea derivatives (compounds **6** and **10–18** and compounds **45–55**) were prepared. For example, the (4-cyanophenyl)thiourea **15** and **52** were prepared from 4-cyanophenyl isothiocyanate (**57**). Both adenosine and xanthine thiourea derivatives containing 4-methyl (**11** and **47**) and 4-methoxy groups (**12** and **48**), and other electron-withdrawing substituents, tended to have diminished receptor affinity. The (4-bromophenyl)thiourea derivatives **16** and **53** were particularly potent at A_1 adenosine receptors.

The degree of irreversible binding was determined after a 45-min incubation of membranes with the putative irreversible ligand, at the indicated concentration. Subsequently, the membranes were exposed to a reversible antagonist, 3-isobutyl-1-methylxanthine, for 16–18 h, which was found to be necessary to remove the unincorporated, high-affinity ligand. Following this prolonged incubation the membranes were washed again and subjected to radioligand [N^6-[(3-[^{125}I]iodo-4-aminophenyl)ethyl]adenosine (^{125}I-APNEA)] binding. Membranes to which no irreversibly bound ligand was added were carried through the whole procedure to serve as appropriate controls. The wash and incubation procedure was shown not to be detrimental to receptor binding. Xanthine isothiocyanate derivatives, compounds **6** and **10** (para- and meta-substituted isomers, respectively), irreversibly inhibited A_1 adenosine receptors in membranes from calf brain and from rat brain in a dose-dependent manner. In calf brain membranes, compound **6** at a concentration of 500 nM (approximately 400-fold greater than the K_i value under these conditions) irreversibly inhibited 90% of the adenosine receptor binding sites, defined by binding of [^{3}H]-XAC. In rat brain at the same concentration, in three separate experiments, the degree of irreversible inactivation of A_1 adenosine receptors was between 62% and 69%. The degree of inactivation of A_1 adenosine receptors by compound **6** was concentration-dependent for both bovine and rat brain (Table I). In five separate experiments, the remaining receptor sites after treatment with p-DITC-XAC at 10^{-7} M (34% of initial density) displayed the same affinity for the radioligand as native receptors (K_D value of 0.5 nM for ^{125}I-APNEA). Thus, the apparent loss of sites is explicable by a complete inactivation of a fraction of the population of receptors. When potent but non-chemically reactive ligands such as compound **4** were used for comparison, the degree of recovery of ^{125}I-APNEA binding sites was nearly total (>90%).

Blocking experiments, in which compound **6** was chemically inactivated prior to receptor binding, were carried out. Compound **6** was dissolved in a minimum of dimethylformamide and treated with a 10-fold excess of ethylenediamine. This isothiocyanate was shown by thin-layer chromatography to be converted completely to the ethylenediamine adduct (**8**, Scheme I) under these conditions. The K_i values against binding of [^{3}H]XAC to rat brain membranes for compounds **6** and **8** were 22 nM and 18 nM, respectively. Although close in affinity to **6**, compound **8** displayed totally reversible binding to A_1 adenosine receptors in rat brain.

The most effective irreversible inhibitors of A_1 adenosine receptors were compounds **10**, **45**, and **46**. The exceptionally potent adenosine aryl isothiocyanate derivatives **45** and **46**, para and meta isomers, respectively, at nanomolar concentration inactivated roughly half of the A_1 receptors. Other purines of intermediate effectiveness as irreversible inhibitors (>20% inhibition at a concentration of 50 nM) were **6**, **19**, **29**, **35**, **40**, **42**, and **56**. Compounds **1**, **26**, **31**, **32**, **38**, and **39** were less potent as irreversible inhibitors. Compounds **34**, **36**, and **37** contained potentially reactive groups, but nevertheless failed to inhibit irreversibly.

K_i values at striatal A_2 adenosine receptors were determined through the inhibition of binding of [^{3}H]-*N*-ethyladenosine-5′-uronamide ([^{3}H]NECA) according to the method of Bruns et al.[18] Those xanthine derivatives evaluated (Table IV) were generally A_1-selective, in the range of 30- to 150-fold. Aryl isothiocyanate derivatives of adenosine, **45** and **46**, were 200- to 600-fold A_1-selective. The suberic acid derivative **35** lost considerable potency at both A_1 and A_2 receptors when two distal methylene groups were substituted by a disulfide linkage (compound **36**). The water-soluble sulfonate derivatives **18** and **55** were nonselective.

Discussion

The choice of chemical groups present in the purine derivatives was made for determination of which nucleo-

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Table IV. Affinity at Striatal A_2 Adenosine Receptors Determined through [^{3}H]NECA Binding Experiments,[18] for Selected Compounds

no.	K_i at A_2 receptors, nM	A_1 selectivity ratio [K_i(at A_2)/K_i(at A_1)]
6	321 ± 79	48.6
10	343 ± 74	144
18	1450 ± 92	3.95
19	105 ± 24	42
26	655 ± 7.7	26.2
29	472 ± 66.6	69
35	207 ± 57	56
36	1050 ± 105	19.1
45	191 ± 54	407
46	176 ± 18.5	203
55	70 ± 6.3	1.37
56	202	76

philic group(s) of the adenosine receptor protein participate(s) in the inhibition reaction. In addition, chain lengths were varied to probe the architecture of the binding site. Lysyl residues, histidyl residues, α-amino groups, and sulfhydryl groups are known to be present in the primary sequence of other membrane-bound receptors (for example, β_2-adrenergic receptors[19]) in the vicinity of the extracellular binding site. Evidence suggestive of a free thiol group present on the A_1 receptor has been reported.[20]

The isothiocyanate group is known to be preferentially reactive with primary and secondary amino groups and thiol groups.[21] Other isothiocyanate-derivatized ligands have been found to be irreversible inhibitors of binding to receptors.[5,7] These probes have found wide application in studying the pharmacology and physiology of opiate and other receptors.

Isothiocyanate derivatives of 1,3-dialkylxanthines (compounds **6**, **10**, and **29**) and of adenosine (compounds **45** and **46**) behave pharmacologically as irreversible inhibitors of central A_1 adenosine receptors. Moreover, adenosine isothiocyanates **45** and **46** are highly selective for A_1 receptors. Xanthine isothiocyanates **6** and **10** are moderately selective for A_1 receptors.

In the xanthine series the position of the isothiocyanate group on the phenyl ring made a substantial difference in the potency of inhibition, with the meta position favored (**10**) over the para position (**6**). In the adenosine series, the meta isomer **46** was a slightly more potent irreversible inhibitor than the *p*-isothiocyanate derivative **45**. Lengthening the chain between the xanthine pharmacophore and the electrophilic group either diminished [(*p*-aminophenyl)acetyl spacer, **26**] or did not affect (glycine spacer, **29**) inhibitory potency.

In a series of amine-reactive *N*-hydroxysuccinimide esters (**1**, **34**, and **35**), only the longest member of the series, compound **35**, substantially inhibited ^{125}I-APNEA binding irreversibly. This is likely due to the relative geometry of the nucleophilic group (likely an amine) relative to the xanthine binding site. Whether the xanthine active ester (**35**), xanthine isothiocyanates (compounds **6**, **10**, and **29**), and adenosine isothiocyanates (compounds **45** and **46**) react with a single nucleophilic residue is unknown. The weak incorporation of thiol reagent **38**, an *N*-alkylmaleimide derivative, is suggestive of a cysteinyl residue in the xanthine binding region.

The identification of irreversible inhibitors of adenosine receptors suggests studies in a number of physiological systems. For example, in the kidney both A_1 and A_2 receptors are present, and these receptors are not readily characterized in competitive binding studies, but might be differentiated with a ligand such as compound **10**.

The central effects of adenosine (evidenced in locomotor depression, analgesia, protection against convulsions) have not been ascribed cleary to either A_1- or A_2-receptor subtypes. Addition of compound **10** as a specific inhibitor in isolated brain preparations or in vivo may prove to be a powerful tool for the study of regulatory effects of endogenous adenosine and delineation of the role of A_1- and A_2-receptor subtypes. It is possible that the metabolism of isothiocyanates in the liver and elsewhere will preclude the entry of purine isothiocyanate derivatives into the brain. If so, it will be necessary to administer the drug intracerebroventricularly for studies of the CNS. The behaviorial effects of metaphit,[5,22] an irreversible isothiocyanate derivative of PCP, have been studied.

The irreversible inhibitors of adenosine receptors have a therapeutic potential. In peripheral sites, a long-acting adenosine antagonist (by virtue of covalent bond formation with the receptor) is of interest as a diuretic or kidney-protective agent, cardiotonic, or stimulator of the immune system. An irreversible adenosine agonist is of interest as a vasodilator, antidiuretic agent, or immunosuppressant.

Materials and Methods

The reported synthesis[11b] of compound 1 has been improved. Disuccinimidyl tartrate (DTT), disuccinimidiyl suberate (DSS), disuccinimidyl dithiobis(propionate) (DSP), and *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS) were purchased from Pierce Chemical Co., Rockford, IL. 1,4-Phenylene diisothiocyanate (DITC) and *N*-succinimidyl 4-maleimidobutyrate (GMBS) were from Fluka, Ronkonoma, NY. 8-[4-[(Carboxymethyl)oxy]-phenyl]-1,3-dipropylxanthine (XCC), 8-[4-[[[[(2-aminoethyl)-amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (XAC, compound 4), and N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]-methyl]anilino]carbonyl]methyl]phenyl]adenosine (ADAC, compound 43) were synthesized as described[10,11b] or obtained from Research Biochemicals, Inc., Natick, MA. Bromoacetic anhydride was obtained from Pfaltz and Bauer (Waterbury, CT). Compounds **6**, **10–20**, **24**, and **33–39** were synthesized by acylation of compound **4**.[11] Compounds **45–56** were synthesized by acylation of compound **43**.[10] Amino acid derivatives of compound **4** were synthesized in the manner described previously,[12] by using the water-soluble 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDAC) in dimethylformamide.

New compounds were characterized by 300-MHz proton NMR (unless noted, chemical shifts are in DMSO-d_6 in ppm from TMS), chemical ionization mass spectroscopy (CIMS, NH_3, Finnigan 1015 spectrometer), and C, H, and N analysis of (±0.4% acceptable).

Certain compounds were characterized by plasma desorption mass spectroscopy[23] and were identified by the presence of positive ion peaks observed at mass = M + 23.

8-[4-[[[(*N*-Succinimidyloxy)carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine (1). 8-[4-[(Carboxymethyl)-oxy]phenyl]-1,3-dipropylxanthine (45 mg, 0.12 mmol), *N*-hydroxysuccinimide (37 mg, 0.32 mmol), and EDAC (64 mg, 0.32 mmol) were combined in 2 mL of DMF. After the mixture had been stirred for 5 min, a solution formed. The solution was cooled in an ice bath, and cold water was added. The resulting precipitate was collected and dried in vacuo: yield 48 mg (86%); characteristic NMR resonances at 8.18 (t, 1 H, NH), 4.42 (t, 1 H, CH, 5.4 Hz),

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3.3 (2 H, CH_2N), 3.26 (s, 6 H, CH_3O) ppm.

8-[4-[[[[(Formylmethyl)amino]carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine (3). 8-[4-[(Carboxymethyl)-oxy]phenyl]-1,3-dipropylxanthine (1.37 g, 3.6 mmol) was condensed with aminoacetaldehyde dimethyl acetal (Aldrich, 0.39 mL, 3.6 mmol) by using dicyclohexylcarbodiimide (0.9 g, 4.4 mmol) in the presence of 1-hydroxybenzotriazole (HOBt; 0.4 g, 3 mmol) to give 8-[4-[[[[[(dimethoxymethyl)methyl]amino]carbonyl]-methyl]oxy]phenyl]-1,3-dipropylxanthine (2). The acetal was then heated at 40 °C with a mixture of 1 N HCl, trifluoroacetic acid, and DMF in a ratio of 1:1:2 to give 3 in 76% yield: characteristic NMR resonances at 9.48 (s, 1 H, CHO), 8.54 (t, 1 H, N H), 4.0 (m, 2 H, CH_2) ppm.

1,3-Dipropyl-8-[4-[[[[[2-[[[(4-isothiocyanatophenyl)-amino]thiocarbonyl]amino]ethyl]amino]carbonyl]-methyl]oxy]phenyl]xanthine (6). 1,4-Phenylene diisothiocyanate (5; 200 mg, 1.0 mmol) was dissolved in a 3 mL of dimethylformamide in a glass container. Compound 4[11] (100 mg, 0.23 mmol) was added in portions with stirring. After 1 h a solution had formed. Dry ether (30 mL) and petroleum ether (30 mL) were added followed by scratching with a glass rod. After overnight incubation in the cold a precipitate formed. The solid was filtered, washed with ether, and dried in vacuo, providing 84 mg (58% yield) of product (6). The solid decomposed at 140 °C, forming another solid which then melts at 220 °C: NMR (DMSO-d_6) δ 9.79 (NH), 8.31 (t, 1 H, NH), 8.07 (d, 2 H, 8-Ar, C-2 and C-6, J = 8.8 Hz), 7.95 (1 H, NH), 7.50 (d, 2 H, NH-Ar, C-3 and C-5, J = 8.8 Hz), 7.33 (d, 2 H, NH-Ar, C-2 and C-6, J = 8.7 Hz), 7.10 (d, 2 H, 8-Ar, C-3 and C-5, = 8.8 Hz), 4.57 (s, 2 H, CH_2O), 4.02 and 3.87 (each t, C-1 Pr), 3.62 (m, 2 H, C-2 Et), 3.4 (2 H, C-1 Et), 1.74 and 1.58 (each m, 2 H, C-2 Pr), 0.89 (q, 6 H, C-3 Pr).

Compound 6 was shown by thin-layer chromatography (silica, chloroform/methanol/acetic acid, 85:10:5) to be quantitatively reactive toward primary amines, such as ethylenediamine, in molar excess, forming 8 (for which were obtained R_f values of 0.87 and 0.33 for 6 and 8, respectively). Compound 6 is stable to storage at −20 °C.

1,3-Phenylene Diisothiocyanate (9). A modification of the procedure of Newman et al.[7] was used. 1,3-Phenylenediamine (Fluka, 0.211 g, 1.95 mmol) was dissolved in 80 mL of chloroform. Water (30 mL) and sodium bicarbonate (0.38 g, 4.5 mmol) were added, and the mixture was stirred vigorously. After 10 min, 0.4 mL (5.2 mmol) of freshly distilled thiophosgene was added. After 1 h the phases were separated, and the organic layer was dried (Na_2SO_4) and evaporated. The residue was triturated with ether/petroleum ether (1:1) and filtered. The filtrate was evaporated, leaving the product, 9, as a white solid (0.30 g, 81% yield), melting at 50.5–51 °C. The NMR spectrum was consistent with the assigned structure. Compound 9 was stable to storage at room temperature for at least several weeks.

1,3-Dipropyl-8-[4-[[[[[2-[[[(4-sulfophenyl)amino]thio-carbonyl]amino]ethyl]amino]carbonyl]methyl]oxy]-phenyl]xanthine Sodium Salt (18). Compound 4 (53.8 mg, 0.126 mmol) was suspended in 2 mL of DMF and treated with 4-sulfophenyl isothiocyanate sodium salt (Fluka, 35 mg, 0.137 mmol). After the mixture was stirred for 2 h, water (4 mL) was added, and the insolubles were filtered and discarded. The filtrate was evaporated on a steam bath. The residue was treated with ether, and the resulting solid was isolated by filtration and recrystallized from DMF/ethyl acetate and then from DMF/methanol/ether. The product, 18, was obtained in 64% yield (53.7 mg): characteristic NMR resonances at 9.68 (s, 1H, ArNHCS), 7.52 (d, 2 H, 8.4 Hz, ortho to S), 7.32 (8.5 Hz, meta to S) ppm.

4-[(*tert*-Butyloxycarbonyl)amino]phenylacetic Acid (22). *p*-Aminophenylacetic acid 21 (1.83 g, 12 mmol) and dibasic sodium phosphate (1.72 g, 12 mmol) were dissolved in 40 mL of H_2O. Methanol (30 mL) and di-*tert*-butyl dicarbonate (2.74 g, 12 mmol) were added. After 2 h, the solution was extracted with ether, which was discarded. The aqueous layer was acidified with 6 N citric acid and extracted three times with ethyl acetate. The organic extracts were combined, dried (Na_2SO_4), and evaporated. The residue was recrystallized from ethyl acetate/petroleum ether to give 1.60 g of the product, 22, mp 141.5–142.5 °C, in 40% yield.

***N*-Succinimidyl 4-[(*tert*-Butyloxycarbonyl)amino]-phenylacetate (23).** Compound 22 (0.42 g, 1.67 mmol) was dissolved in 20 mL of DMF and treated with EDAC (0.6 g, 2 mmol) and *N*-hydroxysuccinimide (0.35 g, 3 mmol). After 2 h ice water was added, resulting in the precipitation of a solid. The product, 23 (yield 0.40 g, 69%, mp 169–170 °C), was dried in vacuo over KOH: characteristic NMR resonances at 9.36 (s, 1 H, NH), 7.42 (d, 2 H, 8.4 Hz), 7.21 (d, 2 H, 8.4 Hz), 2.80 (s, 4 H, succin), 1.47 (s, 9 H, *t*-Bu) ppm.

8-[4-[[[[[2-[[(4-Aminophenyl)acetyl]amino]ethyl]-amino]carbonyl]methyl]oxo]phenyl]-1,3-dipropylxanthine (25). Compound 4 (185 mg, 0.43 mmol) and compound 23 (151 mg, 0.43 mmol) were added to 4 mL of DMF and stirred for 2 h. The mixture was treated with ether to precipitate 8-[4-[[[[[2-[[[4-[(*tert*-butyloxycarbonyl)amino]phenyl]acetyl]-amino]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (24). A 75% yield (0.37 g, mp 215–220 °C) was obtained.

Compound 24 was converted quantitatively to compound 25 upon treatment with neat trifluoroacetic acid for 1 h. The acid was evaporated under a stream of nitrogen and the residue crystallized upon addition of ether. Compound 25 was recrystallized from DMF/methanol/ether and melted at 204–208 °C: characteristic NMR resonances at 6.88 (d, 2 H, 8.1 Hz), 6.47 (d, 2 H, 8.3 Hz), 3.18 (s, 2 H, CH_2Ar) ppm.

8-[4-[[[[[2-[(Bromoacetyl)amino]ethyl]amino]carbonyl]-methyl]oxy]phenyl]-1,3-dipropylxanthine (31). Compound 4 (0.13 g, 0.30 mmol) was suspended in 4 mL of dimethylformamide and treated with bromoacetic anhydride (90 mg, 0.35 mmol). *N*-Methylmorpholine (30 μL, 0.29 mmol) was added slowly. The product, 31, was isolated in 93% yield, mp 275–279 °C.

8-[4-[[[[[2-[[[6-[(*N*-Succinimidyloxy)carbonyl]-*n*-hexyl]carbonyl]amino]ethyl]amino]carbonyl]methyl]-oxy]phenyl]-1,3-dipropylxanthine (35). Compound 4 (58 mg, 0.135 mmol) was suspended in DMF and treated with disuccinimidyl suberate (100 mg, 0.27 mmol). After 1 h the product was isolated as a precipitate in 92% yield upon addition of ether and petroleum ether. The product, 35, a white solid melting at 189–197 °C, was recrystallized from DMF/water.

***N*^{6}-[4-[[[[4-(Carboxymethyl)phenyl]amino]carbonyl]-methyl]phenyl]adenosine (41).** *N*^{6}-[4-[[[[4-[[(Methyloxy)-carbonyl]methyl]phenyl]amino]carbonyl]methyl]phenyl]-adenosine[10a] (1.48 g, 2.7 mmol) was dissolved in 10 mL of dimethylformamide and treated with 1 mL of 4 M NaOH. After 24 h, the solution was acidified with 5 mL of 2 M citric acid and then treated with saturated sodium chloride (5 mL) and water (10 mL). The precipitate was collected by centrifugation and washed with water (3×), a small amount of cold methanol (2×), and ether: yield 1.24 g (42 %); mp 182–184 °C.

***N*^{6}-[4-[[[[4-[[(*N*-Succinimidyloxy)carbonyl]methyl]-phenyl]amino]carbonyl]methyl]phenyl]adenosine (42).** Compound 41 (1.08 g, 2.0 mmol) was dissolved in 10 mL of dimethylformamide and treated with EDAC (2.5 g) and *N*-hydroxysuccinimide (2.4 g). After 1 h, the reaction was complete as judged by using thin-layer chromatography (silica, chloroform/methanol/acetic acid, 85:10:5). The product (R_f values for acid and ester, 0.32 and 0.40, respectively) precipitated slowly following cooling in an ice bath and addition of saturated sodium chloride. The product was isolated by centrifugation and washed with water, methanol, and ether. The product did not melt sharply, but decomposed at 175 °C; yield 0.95 g (74%). The IR spectrum shows an intense ester carbonyl peak at 1740 cm^{-1}.

***N*^{6}-[4-[[[4-[[[(2-Isothiocyanatoethyl)amino]carbonyl]-methyl]anilino]carbonyl]methyl]phenyl]adenosine (44).** Compound 43 (25 mg, 43 μmol) and triethylamine (6 μL) were dissolved in 1 mL of DMF with warming. After cooling in an ice bath, distilled thiophosgene (3.9 μL, 51 μmol) was added with stirring. Water and saturated NaCl were added to obtain a white precipitate, which was dried in vacuo over NaOH; yield 13 mg (48%) of product, 44. IR peaks appeared at 3460, 2120, 1640, 1600, 1515, 1480, 1420, 1100, 1060, and 1030 cm^{-1}. Thin-layer chromatography and the Cf plasma desorption spectrum[23] was consistent with the assigned structure (95% maximum peak at mass 641 corresponding to M + Na^+) and showed no evidence of residual 43.

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N^6-[4-[[[4-[[[[2-[[[(3-Isothiocyanatophenyl)amino]thiocarbonyl]amino]ethyl]amino]carbonyl]methyl]anilino]carbonyl]methyl]phenyl]adenosine (**45**). Compound **43** (19.3 mg, 34 μmol) was suspended in 1 mL of DMF with stirring and treated with 1,3-phenylene diisothiocyanate (**9**; 14 mg, 71 μmol). After 1 h the solution was treated with dry ether to precipitate the product (20 mg, 78% yield, mp 182–182 °C), **45**: NMR (Me_2SO-d_6) δ 9.80 (s, 1 H, ArNHCS), 10.08 and 9.92 (each s), 8.54 and 8.39 (each s, adenine), 7.84 (d, 2 H, J = 8.3 Hz, ortho to adenine NH), 7.59 (s, 1 H, ortho to ArNCS), 7.50 (d, 2 H, J = 7.6 Hz, ortho to NHCO), 7.34 (m, 2 H, ArNCS), 7.28 (d, 2 H, J = 8.3 Hz, meta to adenine NH), 7.17 (d, 2 H, J = 7.7 Hz, meta to NHCO), 7.1 (1 H, ArNCS), 5.94 (d, 1 H, ribose C_1, J = 5.9 Hz), 5.47 (d, 1 H, OH, J = 6.0 Hz), 5.28 (t, 1 H, OH, J = 5 Hz), 5.21 (d, 1 H, OH, J = 3.6 Hz), 4.63, 4.17, and 3.97 (each m, 1 H, ribose CHO), 3.67 and 3.54 (m, 2 H, CH_2OH), 3.59 (s, 4 H, CH_2Ar), 3.2–3.4 (m, 4 H, NCH_2CH_2N).

N^6-[4-[[[4-[[[[2-[[[(4-Sulfophenyl)amino]thiocarbonyl]amino]ethyl]amino]carbonyl]methyl]anilino]carbonyl]methyl]phenyl]adenosine Sodium Salt (**55**). Compound **43** (17.5 mg, 30 μmol) was suspended in 1 mL of DMF with stirring and treated with 4-sulfophenyl isothiocyanate sodium salt (10 mg, 40 μmol). After overnight incubation with stirring a solution formed. The reaction was filtered and treated with dry ether to precipitate an oil. The oil was treated with methanol to obtain the product (13 mg, 51% yield, mp 219–229 °C), **55**: characteristic NMR resonances at 9.65 (s, 1 H, ArNHCS), 7.49 (d, 2 H, 8.3 Hz, ortho to S), 7.29 (d, 2 H, 8 Hz, meta to S).

N^6-[4-[[[4-[[[[2-[(Bromoacetyl)amino]ethyl]amino]carbonyl]methyl]anilino]carbonyl]methyl]phenyl]adenosine (**56**). Compound **43** (16 mg, 28 μmol) was suspended in 1 mL of DMF with stirring. Bromoacetic anhydride (50 mg) was added as a solid, and the mixture was stirred for 5 min. Ether (4 mL) was added to precipitate the product, **36**, which was collected by filtration and dried in vacuo over KOH. Yield was 17.6 mg (90.9%) of **56**, a white solid that decomposed at 220 °C without melting: characteristic NMR resonance at 3.82 (s, 2 H, CH_2Br) ppm. The Cf plasma desorption MS[23] showed peaks corresponding to M + H^+, M + Na^+, and loss of ribose.

Biochemical Assays. Stock solutions of xanthines in the millimolar concentration range in dimethyl sulfoxide were prepared for binding assays and stored frozen. Solutions were warmed to 50 °C prior to successive dilution in DMSO (approximately 50-fold) and then in aqueous medium. Solutions of compound **10** at <4 mg/mL could be obtained by dissolving in DMSO (40 mg/mL) and adding a 40% aqueous solution of γ-(hydroxypropyl)cyclodextrin[24] with warming. Aryl isothiocyanates were unstable at pH >10.

Competitive Binding Assay Using [^{3}H]PIA. Inhibition of binding of 1 nM [^{3}H]-N^6-(phenylisopropyl)adenosine (Du Pont New England Nuclear, Boston, MA) to A_1 adenosine receptors in rat cerebral cortex membranes was assayed as described.[11] Inhibition of binding by a range of concentrations of a xanthine or adenosine derivative was assessed in triplicate in at least three separate experiments. IC_{50} values were converted to K_i values by using a K_D value for [^{3}H]PIA of 1.0 nM and the Cheng–Prusoff equation.[25]

Competitive Binding Assay and Incorporation Studies Using ^{125}I-APNEA. Binding assays in bovine brain and in rat brain to examine irreversible incorporation utilized ^{125}I-APNEA, which was synthesized as described previously.[9a] Sodium [^{125}I]iodide was obtained from Amersham (Arlington Heights, IL).

Rat cerebral cortex and bovine cerebral cortex membranes were prepared as described previously.[26] Membranes were treated with adenosine deaminase (0.5 unit/mL) for 20 min at 37 °C prior to radioligand binding studies or incorporation studies.

Membranes (40 μg, 150 μL) were incubated for 1 h at 37 °C in a total volume of 250 μL, containing 50 μL of radioligand of the indicated concentration and 50 μL of the competing ligand. Isothiocyanates and other chemically reactive derivatives were weighed out prior to use and dissolved in DMSO. The DMSO solutions were diluted to a concentration of less than 0.1 mM prior to adding to aqueous medium. Bound and free radioligand was separated by addition of 4 mL of a solution containing 50 mM Tris-HCl, 10 mM magnesium chloride, and 1 mM EDTA at pH 8.26 (buffer A) with 0.02% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) at 5 °C followed by vacuum filtration on glass filters with additional washes totaling 12 mL of buffer. Filters were counted in a γ counter at an efficiency of 75%. Percent inhibition of binding was determined through the use of full saturation curves using ^{125}I-APNEA with concentrations ranging from 0.1 to 2.5 nM. Nonspecific binding was determined with 10^{-5} M (*R*)-PIA.

Saturation was analyzed by use of computer modeling programs as described previously.[27]

For studies of irreversible incorporation, membranes were prepared as described above and then incubated with the indicated (Tables I and II) concentration of ligand for 45 min at 37 °C. Initial experiments demonstrated that complete incorporation had occurred by 30 min (data not shown). Membranes were then washed three times by sequential resuspension and centrifugations with buffer A containing 0.02% Chaps. Membranes were then suspended in buffer A containing 10^{-4} M 3-isobutyl-1-methylxanthine (IBMX) and incubated at 25 °C for 18 h with shaking. Membranes were then washed twice with buffer A, treated with adenosine deaminase as described above, and used in radioligand binding studies as described above.

The long treatment with IBMX was found necessary to remove all of the nonincorporated ligands from membranes. Multiple washes (up to eight) with buffer alone were insufficient to remove all the reversibly bound ligands.

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