

macromolecules. Attached V79 cells were cultured in 60-mm plastic culture dishes, to a density of about 1×10^5 cells/cm² at which time the cells were undergoing a logarithmic rate of growth. Growth medium was then replaced with fresh medium containing [³H]TdR ([methyl,1',2'-³H]thymidine: 95 Ci/mmol, 2 μ Ci/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% perchloric 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 ³H 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 [³H]TdR and experimental cells labeled with [¹⁴C]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 ³H and ¹⁴C 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 ¹⁴C dpm) eluting prior to the DNA from untreated control cells (proportional to the ³H 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 A₁ Adenosine Receptors

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Functionalized congeners derived from 1,3-dipropyl-8-phenylxanthine and from N⁶-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 A₁ 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 A₁ 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 A₁-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 A₁ or A₂ 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

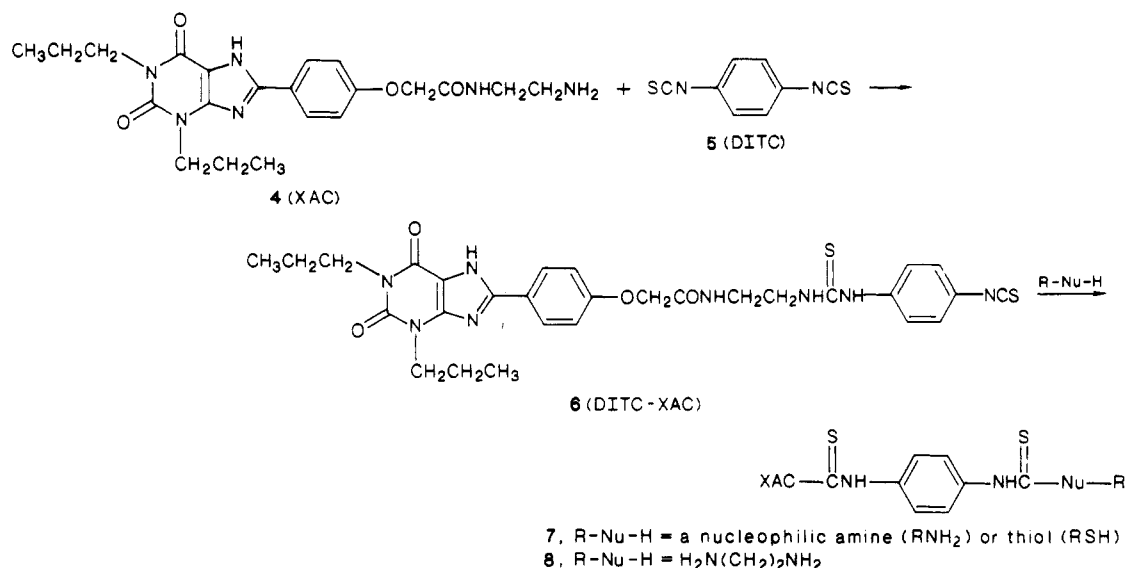
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Scheme I



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^{16,9} have hitherto been described. We now report a set of reactive adenosine receptor ligands, derived from agonist¹⁰ and antagonist¹¹ 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 N⁶-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 (N⁶-phenyl-adenosine 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 A₁ selective in reversing cardiac depressant effects of adenosine agonists¹⁴ and in renin release¹³ and as a radioligand having nanomolar affinity.¹¹ N⁶-[4-[[[(2-Aminoethyl)amino]carbonyl]methyl]anilino]carbonyl]methyl]-phenyladenosine (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 4¹¹ 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 A₁ 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

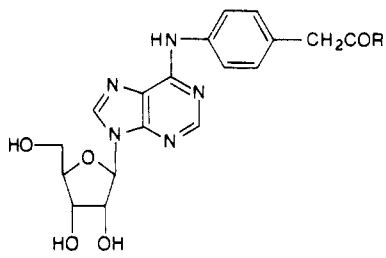
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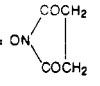
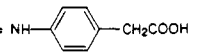
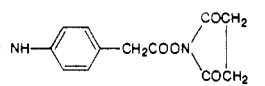
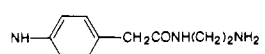
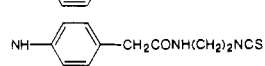
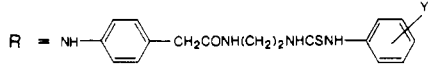
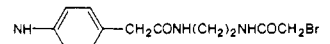
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Table I. Xanthine Derivatives

compd no. and structure	K _i at central A ₁ receptors, nM		
	rat ^a	cal ^b	% inhibn (concn) ^c
1, R =	9.0 ± 0.7 ^d	e	10 (50)
3, R = NHCH ₂ CHO	266 ± 40 ^d	3.17 ± 0.4	0 (500)
4, R' = H	R = NH(CH ₂) ₂ NHR' 1.1 ± 0.05	0.3 ± 0.05	e
	R' = CSNH-		
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 ₃ Na	367 ± 73.4	e	e
19, R' = SO ₂ -	2.5 ± 0.47	e	23 (50)
20, R' = SO ₂ -	72 ± 7.2 ^d	e	e
24, R' = COCH ₂ -	53 ± 12	e	e
25, R' = COCH ₂ -	40	e	e
26, R' = COCH ₂ -	25.0 ± 6.6	e	20 (50)
29, R' = COCH ₂ NHCSNH-	6.85 ± 1.14	e	50 (50)
30, R' = CO(CH ₂) ₄ COCH ₃	44 ± 9	6.2 ± 0.7	0 (500)
31, R' = COCH ₂ Br	17.6 ± 1.5 ^d	e	16 (50)
32, R' = CO-	11.5 ± 1.5 ^d	10.1 ± 1.2	15 (50)
34, R' = CO(CH ₂) ₂ COON-	20.0 ± 4.5	e	0 (500)
35, R' = CO(CH ₂) ₆ COON-	3.69 ± 0.71	23.0 ± 2.0	32 (50)
36, R' = CO(CH ₂) ₂ SS(CH ₂) ₂ COON-	54.9 ± 8.6	e	0 (50)
37, R' = CO(CH ₂) ₂ SS-	23.8 ± 3.51	e	3 (50)
38, R' = CO(CH ₂) ₃ N-	37.8 ± 4.38	e	15 (50)
39, R' = COCH ₂ NHCO(CH ₂) ₃ N-	23.2 ± 2.14	e	9 (50)

^a Against [³H]PIA, ±SEM; for compounds that inhibit irreversibly, the value represents an apparent K_i, since a fraction of the sites are inactivated. ^b Against [¹²⁵I]-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 [¹²⁵I]-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
			
45, Y = 4-NCS	0.469 ± 0.13	d	39 (1)
46, Y = 3-NCS	0.867 ± 0.114	d	>80 (10)
47, Y = 4-CH ₃	3.72 ± 0.44	d	62 (1)
48, Y = 4-OCH ₃	8.52 ± 0.85	d	>90 (10)
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 ₃ Na	51.1 ± 7.81	d	d
56, R = 	8.30 ± 0.45	d	38 (50)

^a Against [³H]PIA, ±SEM; for compounds that inhibit irreversibly, the value represents an apparent K_i , since a fraction of the sites are inactivated. ^b Against [¹²⁵I]-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 [¹²⁵I]-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*-butoxy-carbonyl)-*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 ₅ O ₆	C, H, N
3	B	76	203–206	C ₂₁ H ₂₅ N ₅ O ₅	d
6	C	58	140 ^d	C ₂₉ H ₃₂ N ₈ O ₄ S ₂ ·H ₂ O	C, H, N
9	D	81	50.5–51	C ₈ H ₄ N ₂ S ₂	C, H, N
10	C	62	186–188	C ₂₉ H ₃₂ N ₈ O ₄ S ₂ ·0.5H ₂ O	C, H, N
11	C	87	205–208	C ₂₉ H ₃₅ N ₇ O ₄ S	C, H, N
12	C	89	170–174	C ₂₉ H ₃₅ N ₇ O ₅ S·H ₂ O	C, H, N
13	C	89	163–165	C ₂₈ H ₃₂ N ₇ O ₄ FS	C; H, N ^c
14	C	58	257–265	C ₂₈ H ₂₈ N ₇ O ₄ F ₅ ·0.5H ₂ O	C, H, N
15	C	84	249–251	C ₂₉ H ₃₂ N ₈ O ₄ S	C, H, N
16	C	70	205–208	C ₂₈ H ₃₂ N ₇ O ₄ BrS	C, H, N
17	C	93	248–250	C ₂₈ H ₃₂ N ₈ O ₆ S·0.5H ₂ O	C, H, N
18 ^b	C	64	275 ^d	C ₂₈ H ₃₂ N ₇ O ₇ S ₂ Na·2H ₂ O	H; C, N ^c
19	E	57	256–260	C ₂₇ H ₃₁ N ₆ O ₆ S ₂ Cl	e
20	E	90	211–216	C ₂₈ H ₃₄ N ₆ O ₆ S·0.5H ₂ O	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 ₂ O ₆	C, H, N
24	G	75	215–220	C ₃₄ H ₄₃ N ₇ O ₇ ·0.5H ₂ O	C, H, N
25	B	89	204–208	C ₂₉ H ₃₅ N ₇ O ₅ ·2H ₂ O	C, H, N
26 ^b	C	50	194–204	C ₃₇ H ₃₉ N ₉ O ₅ S ₂ ·H ₂ O	C, H, N
27	A	81	210–212	C ₂₈ H ₃₉ N ₇ O ₇ ·H ₂ O	C, H, N
28	B	100	200–210	C ₂₆ H ₃₂ N ₇ O ₇ F ₃ ·DMF	H, N; C ^c
29	C	90	173–175	C ₃₁ H ₃₅ N ₉ O ₅ S ₂ ·H ₂ O	C, H; N ^c
30	A	33	236–239	C ₂₈ H ₃₈ N ₆ O ₆	C, H, N
31	F	93	275–279	C ₂₃ H ₂₉ N ₆ O ₅ Br·H ₂ O	C, H, N
32 ^b	G	78	277–280	C ₂₉ H ₃₃ N ₆ O ₅ Br	f
33	F	80	210–212	C ₂₅ H ₃₂ N ₆ O ₇ ·1.5H ₂ O	C, H, N
34	A	83	161–162	C ₂₉ H ₃₅ N ₇ O ₉ ·H ₂ O	C, H, N
35	G	92	189–197	C ₃₃ H ₄₃ N ₇ O ₉ ·DMF	C, H, N
36	G	46	219–224	C ₃₁ H ₃₉ N ₇ O ₉ S ₂ ·2DMF	H, N; C ^c
37	G	83	212–219	C ₂₉ H ₃₅ N ₇ O ₅ S ₂	C, H, N
38	G	74	203–206	C ₂₉ H ₃₅ N ₇ O ₇ ·0.5H ₂ O	C, H, N
39	G	70	192–195	C ₃₁ H ₃₉ N ₈ O ₈ ·0.5H ₂ O	C, H, N
40	A	41	132–135	C ₂₂ H ₂₂ N ₆ O ₈ ·1.5H ₂ O	C, H, N
41	H	86	182–184	C ₂₆ H ₂₆ N ₆ O ₇ ·1.5H ₂ O	C, H, N
42	A	74	175 ^d	C ₃₀ H ₂₉ N ₇ O ₉	d
44 ^b	D	48		C ₂₉ H ₃₀ N ₈ O ₆ S	d
45	C	77	179–181	C ₃₆ H ₃₆ N ₁₀ O ₆ S ₂ ·H ₂ O	C, H, N
46	C	78	182–183	C ₃₆ H ₃₆ N ₁₀ O ₆ S ₂	C, H, N
47	C	90	178–182	C ₃₆ H ₃₉ N ₉ O ₆ S·0.5H ₂ O	C, H, N
48	C	95	171–174	C ₃₆ H ₃₉ N ₉ O ₇ S·H ₂ O	C, H, N
49	C	91	171–174	C ₃₅ H ₃₆ N ₉ O ₆ FS·2H ₂ O	C, H, N
50	C	63	169–174	C ₃₅ H ₃₆ N ₉ O ₆ FS·2H ₂ O·2DMF	C, H, N
51 ^b	C	40		C ₃₅ H ₃₂ N ₉ O ₆ F ₅ S·0.5H ₂ O	C, H, N
52	C	96	171–173	C ₃₆ H ₃₆ N ₁₀ O ₆ S	C, H, N
53	C	87	178–182	C ₃₅ H ₃₆ N ₉ O ₆ BrS	C, H, N
54	C	99	172–175	C ₃₅ H ₃₆ N ₁₀ O ₈ S·0.5H ₂ O	C, H, N
55 ^b	C	51	219–229	C ₃₅ H ₃₆ N ₉ O ₉ S ₂ Na·H ₂ O	C, H, N
56 ^b	F	91	dec 220	C ₃₀ H ₃₃ N ₈ O ₇ Br·H ₂ O	C, H, N
57	D	93	118–120	C ₈ H ₄ N ₂ S	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 H₂S 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, C₂Ar, *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, CH₂Ar) 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.

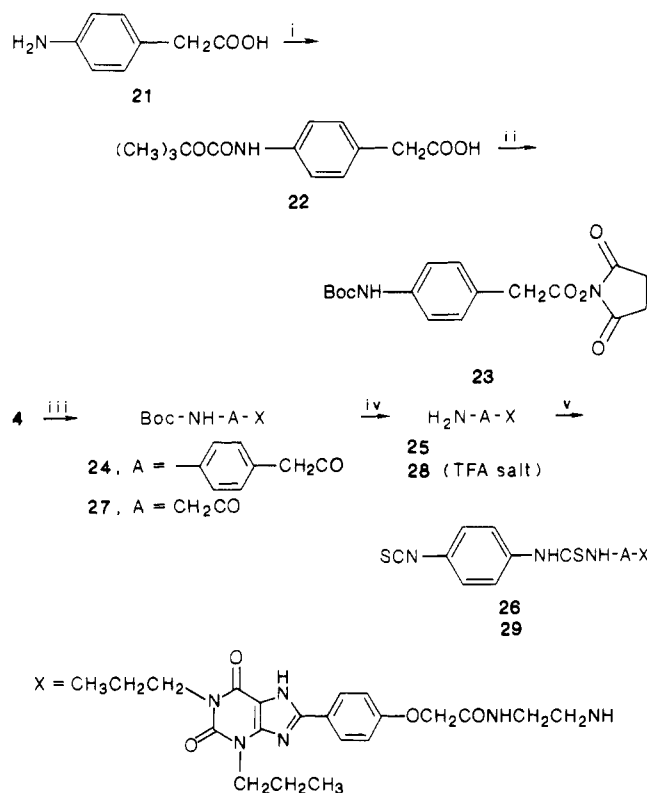
The reaction of compound 4 with the homobifunctional reagents disuccinimidyl tartrate and *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester yielded predominantly side products, and the desired conjugates were not isolated.

Biology. The xanthine and adenosine derivatives were assayed for affinity at A₁ adenosine receptors in rat brain membranes (Tables I and II), by use of [³H]-*N*⁶-(phenylisopropyl)adenosine [(*R*)-[³H]PIA] as a radioligand, and were found to have high affinity for this class of receptors. The affinity for A₁ 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*-toluene-sulfonamide 20. The suberoyl active ester 35 was 5.4-fold more potent than the smaller, corresponding succinoyl derivative 34.

(17) Ukena, D.; Jacobson, K. A.; Padgett, W.; Ayala, C.; Shamim, M. T.; Kirk, K. L.; Daly, J. W. *FEBS Lett.* 1986, 209, 122.

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 A₁ 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-[¹²⁵I]iodo-4-aminophenyl)ethyl]adenosine ([¹²⁵I]-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 A₁ 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 [³H]-XAC. In rat brain at the same concentration, in three separate experiments, the degree of irreversible inactivation of A₁ adenosine receptors was between 62% and 69%. The degree of inactivation of A₁ 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 [¹²⁵I]-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 [¹²⁵I]-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 [³H]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 A₁ adenosine receptors in rat brain.

The most effective irreversible inhibitors of A₁ 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 A₁ 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 A₂ adenosine receptors were determined through the inhibition of binding of [³H]-*N*-ethyladenosine-5'-uronamide ([³H]NECA) according to the method of Bruns et al.¹⁸ Those xanthine derivatives evaluated (Table IV) were generally A₁-selective, in the range of 30- to 150-fold. Aryl isothiocyanate derivatives of adenosine, 45 and 46, were 200- to 600-fold A₁-selective. The suberic acid derivative 35 lost considerable potency at both A₁ and A₂ 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-

(18) Bruns, R. F.; Lu, G. H.; Pugsley, T. A. *Mol. Pharmacol.* 1986, 29, 331–346.

Table IV. Affinity at Striatal A₂ Adenosine Receptors Determined through [³H]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 receptors¹⁹) in the vicinity of the extracellular binding site. Evidence suggestive of a free thiol group present on the A₁ 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 A₁ adenosine receptors. Moreover, adenosine isothiocyanates 45 and 46 are highly selective for A₁ receptors. Xanthine isothiocyanates 6 and 10 are moderately selective for A₁ 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 [¹²⁵I]-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 A₁ and A₂ 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 clearly to either A₁- or A₂-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 A₁- and A₂-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 behavioral 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), disuccinimidyl suberate (DSS), disuccinimidyl dithiobis(propionate) (DSP), and *m*-maleimido-benzoyl *N*-hydroxysuccinimide ester (MBS) were purchased from Pierce Chemical Co., Rockford, IL. 1,4-Phenylene diisothiocyanate (DITC) and *N*-succinimidyl 4-maleimidobutylate (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, NH₃, Finnigan 1015 spectrometer), and C, H, and N analysis of (±0.4% acceptable).

Certain compounds were characterized by plasma desorption mass spectroscopy²³ 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, CH₂N), 3.26 (s, 6 H, CH₃O) 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, CH₂) 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 4¹¹ (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, CH₂O), 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 (Na₂SO₄) 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-sulfo)phenyl]amino]thiocarbonyl]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-sulfo)phenyl 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 H₂O. 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 (Na₂SO₄), 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, CH₂Ar) 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-[(*N*-Succinimidyl)oxy]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*-Succinimidyl)oxy]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 spectrum²³ 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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N⁶-[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 (Me₂SO-*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 C₁, *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, CH₂OH), 3.59 (s, 4 H, CH₂Ar), 3.2–3.4 (m, 4 H, NCH₂CH₂N).

N⁶-[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).

N⁶-[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, CH₂Br) ppm. The Cf plasma desorption MS²³ 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)cyclodextrin²⁴ with warming. Aryl isothiocyanates were unstable at pH >10.

Competitive Binding Assay Using [³H]PIA. Inhibition of binding of 1 nM [³H]-N⁶-(phenylisopropyl)adenosine (Du Pont New England Nuclear, Boston, MA) to A₁ 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. IC₅₀ values were converted to K_i values by using a K_D value for [³H]PIA of 1.0 nM and the Cheng-Prusoff equation.²⁵

Competitive Binding Assay and Incorporation Studies Using ¹²⁵I-APNEA. Binding assays in bovine brain and in rat brain to examine irreversible incorporation utilized ¹²⁵I-APNEA, which was synthesized as described previously.^{9a} Sodium [¹²⁵I]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 ¹²⁵I-APNEA with concentrations ranging from 0.1 to 2.5 nM. Nonspecific binding was determined with 10⁻⁶ 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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