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Trifunctional Agents as a Design Strategy for Tailoring Ligand Properties: Irreversible Inhibitors of A₁ Adenosine Receptors†

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Abstract

The 1,3-phenylene diisothiocyanate conjugate of XAC (8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]-oxy]phenyl]-1,3-dipropylxanthine, a potent A₁ selective adenosine antagonist) has been characterized as an irreversible inhibitor of A₁ adenosine receptors. To further extend this work, a series of analogues were prepared containing a third substituent in the phenyl isothiocyanate ring, incorporated to modify the physiochemical or spectroscopic properties of the conjugate. Symmetrical trifunctional cross-linking reagents bearing two isothiocyanate groups were prepared as general intermediates for cross-linking functionalized congeners and receptors. Xanthine isothiocyanate derivatives containing hydrophilic, fluorescent, or reactive substituents, linked via an amide, thiourea, or methylene group in the 5-position, were synthesized and found to be irreversible inhibitors of A₁ adenosine receptors. The effects of the 5-substituent on water solubility and on the A₁/A₂ selectivity ratio derived from binding assays in rat brain membranes were examined. Inhibition of binding of [³H]-N⁶-(2-phenylisopropyl)-adenosine and [³H]CGS21680 (2-[[2-[4-(2-carboxyethyl)phenyl]ethyl]amino]adenosine-5'-N-ethylcarboxamide) at central A₁ and A₂ adenosine receptors, respectively, was measured. A conjugate of XAC and 1,3,5-triisothiocyanatobenzene was 894-fold selective for A₁ receptors. Reporter groups, such as fluorescent dyes and a spin-label, were included as chain substituents in the irreversibly binding analogues, which were designed for spectroscopic assays, histochemical characterization, and biochemical characterization of the receptor protein.

Adenosine acts as a neuromodulator at two receptor subtypes, A₁ and A₂, which in general inhibit or stimulate adenylate cyclase, respectively (1, 2). Selective agonists (3, 4) and antagonists (5, 6) of each receptor subtype have been reported. The high-affinity, A₁-selective antagonist XAC1 (**1**), a 1,3-dipropylxanthine derivative that contains an amine-functionalized chain, was developed by using a functionalized congener approach (5). XAC having a K_i value at rat brain A₁ receptors of 1.2 nM has been useful in characterizing the A₁ adenosine receptor both as a radioligand (7) and as a ligand immobilized via its alkyl amino group for affinity chromatography of the receptor protein (8, 9). The *m*- and *p*-phenylene diisothiocyanate conjugates of XAC [*m*-DITC-XAC2(**2**) and *p*-DITC-XAC3(**3**)],

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¹8-[4-[[[(2-Aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine.

²8-[4-[[[(2-Isithiocyanatophenyl)amino](thiocarbonyl)]-amino]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine.

were prepared as high-affinity, irreversible A₁ antagonists, with apparent K_i values at rat brain A₁ receptors of 2.4 and 6.6 nM, respectively (10, 11). These chemically reactive ligands have been used for identifying the A₁ receptor on electrophoretic gels (11) and for blocking A₁ receptor mediated effects in physiological studies (12).

1: R = H

A general approach to systematically modifying the structure of irreversible ligand derivatives was desired to provide analogues with specific properties. In previous studies of reversible ligands bearing spectroscopic labels (13), only moderate receptor affinities were attained, and this limited the utility of the ligands for histochemical studies. A spectroscopic probe that binds irreversibly to the receptor would preclude the problems of rapid washout and long equilibration times associated with reversible ligands. Another difficulty encountered with 8-phenylxanthine derivatives in physiological studies (12) is their typically low water solubility (14). In this study, we explore a conceptually broad synthetic methodology that permits the incorporation of a reporter group (spin-label, radio-ligand, fluorescent, etc.) or of a group for altering physical properties, such as water solubility, into an irreversible inhibitor.

Figure 1 presents an overview of our trifunctional strategy. The essential feature of this plan is a cross-linking reagent (**4**), a 1,3,5-substituted benzene that includes three potentially reactive sites. These sites are (1) an acylating group for reaction with a functionalized congener, in this case the acylating group consisting of an isothiocyanate group to react with the primary amino group of xanthine **1** to afford general structure **5**; (2) another chemically reactive electrophilic group, such as an isothiocyanate, to combine irreversibly with the receptor protein; and (3) a site for incorporating a solubilizing or reporter moiety (group R' in Figure 1).

EXPERIMENTAL PROCEDURES

General Procedures

New compounds were characterized (and resonances assigned) by 300-MHz proton nuclear magnetic resonance spectroscopy using a Varian XL-300 FT-NMR spectrometer. The assignments, for a typical xanthine derivative, are specified below for compound **5m**. Unless noted, chemical shifts are expressed as ppm downfield from tetramethylsilane. Synthetic intermediates were characterized by ¹H NMR and by chemical ionization mass spectroscopy (CIMS, NH₃) using a Finnigan 1015 mass spectrometer modified with EXTREL electronics or on a Finnigan 4500 MS. Accurate mass (using fast atom bombardment) was measured on

³8-[4-[[[2-[[[4-(4-Isothiocyanatophenyl)amino](thiocarbonyl)]-amino]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine.

a JEOL SX102 high-resolution mass spectrometer. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. C, H, and N analysis was carried out by Atlantic Microlabs (± 0.4 % acceptable). The Chromatotron is a radially accelerated thin-layer chromatography device manufactured by Harrison Research, Palo Alto, CA. [^3H]- N^6 -(Phenylisopropyl)adenosine and [^3H]-5'-(*N*-ethylcarbamoyl)adenosine were from Du Pont NEN Products, Boston, MA. N^6 -Cyclopentyladenosine and XAC (**1**) were obtained from Research Biochemicals, Inc., Natick, MA. 3,5-Dinitroaniline and 2,6-diaminopyridine were obtained from Lancaster Synthesis (Windham, NH). 3,5-Dinitrobenzoyl chloride and 3,5-dinitrobenzamide were obtained from Aldrich Chemical Co. (Milwaukee, WI). 4-[[4-(Fluoromethyl)benzoyl]amino]-butylamine was synthesized as described (15). 5-[(2-Aminoethyl) thioureydyl]fluorescein was obtained from Molecular Probes, Inc. (Eugene, OR).

General Method for Coupling XAC with Diisothiocyanates

XAC (25 mg, 58.4 μM) was suspended in 0.5 mL of dimethylformamide, and a 3 molar excess of the requisite diisothiocyanate was added. This was sonicated for 3–5 min, during which time complete dissolution occurred. The reaction was monitored by HPLC (UV detection at 254 nm) for disappearance of XAC and was complete within 5 min. With a gradient mixture of 20–60% acetonitrile and water containing 0.1% TFA⁴ over 20 min, the retention time for XAC was 4 min (Altex Ultrasphere ODS column, 4.6 mm $\times$ 25 cm). The conjugates were retained much longer than XAC (roughly 20 min). After XAC had disappeared from the reaction, either ethyl acetate or ether was added to precipitate the product. This precipitate was collected by centrifugation and was washed three times with fresh ether. The solid material was then dried overnight in vacuo at 50 $^{\circ}\text{C}$.

8-[4-[[[2-[[[5-(Ethoxycarbonyl)-3-isothiocyanatophenyl] (thiocarbonyl)]amino]ethyl]amino]carbonyl] methyl]oxy]phenyl]-1,3-dipropylxanthine (5a)—was synthesized by the above method from compound **26**. The product was homogeneous by TLC (R_f = 0.73, silica, chloroform/methanol/acetic acid 85/10/5; NMR (DMSO- d_6) δ 8.30 (s, 1 H), 8.05 (d, 2 H, J = 8.5 Hz), 7.92 (s, 2 H), 7.55 (s, 1 H), 7.08 (d, 2 H, J = 8.5 Hz), 4.57 (s, 2 H), 4.30 (q, 2 H), 4.00 and 3.86 (each m, 2 H), 1.75 and 1.57 (each m, 2 H), 1.30 (t, 3 H), 0.90 (m, 6 H) ppm.

8-[4-[[[2-[[[5-(Hydroxymethyl)-3-isothiocyanatophenyl] (thiocarbonyl)]amino]ethyl]amino]carbonyl] methyl]oxy]phenyl]-1,3-dipropylxanthine (5b)—was synthesized by the above method from compound **28**: NMR (DMSO- d_6) δ 8.31 (m, 1 H), 8.07 (d, 2 H, J = 8.7 Hz), 7.48 (s, 1 H), 7.24 (s, 1 H), 7.10 (d, 2 H, J = 8.7 Hz), 7.06 (s, 1 H), 4.58 (s, 2 H), 4.47 (s, 2 H), 4.01 and 3.86 (each m, 2 H), 1.74 and 1.58 (each m, 2 H), 0.90 (m, 6 H) ppm.

The following compounds were synthesized by the above method from structure **14** or **19**.

8-[4-[[[2-[[[5-(Aminocarbonyl)-3-isothiocyanatophenyl] (thiocarbonyl)]amino]ethyl]amino]carbonyl] methyl]oxy]phenyl]-1,3-dipropylxanthine (5c)—mp >250 $^{\circ}\text{C}$; NMR (DMSO- d_6) δ 8.33 (m, 1 H), 8.06 (m, 3 H), 7.75, 7.61, and 7.55 (each s, 1 H), 7.10 (d, 2 H, J = 8.8 Hz), 4.58 (s, 2 H), 4.01 and 3.87 (each m, 2 H), 1.75 and 1.58 (each m, 2 H), 0.90 (m, 6 H) ppm. Accurate mass (FAB) consistent with assigned structure.

⁴Trifluoroacetic acid.

⁵Dimethyl sulfoxide.

8-[4-[[[[[2-[[[5-[[[2-(Dimethylamino)ethyl]amino]-carbonyl]-3-isothiocyanatophenyl](thiocarbonyl)]-amino]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (5d)—mp >250 °C; NMR (DMSO-*d*₆) δ 8.35 (m, 1 H), 8.05 (d, 2 H, *J* = 8.6 Hz), 7.85-7.60 (m, 3 H), 7.10 (d, 2 H, *J* = 8.6 Hz), 4.60 (s, 2 H), 4.0 (m, 4 H), 3.85 (m, 4 H), 2.50 (s, 6 H), 1.74 and 1.58 (each m, 2 H), 0.90 (m, 6 H) ppm.

8-[4-[[[[[2-[[[5-[[[2-(Acetylamino)ethyl]amino]carbonyl]-3-isothiocyanatophenyl](thiocarbonyl)]-amino]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (5e)—mp >250 °C; NMR (DMSO-*d*₆) δ 8.55 (m, 1 H), 8.05 (d, 2 H, *J* = 8.4 Hz), 7.90 (s, 2 H), 7.80 (s, 1 H), 7.08 (d, 2 H, *J* = 8.4 Hz), 4.50 (s, 2 H), 4.0 (m, 4 H), 3.80 (m, 4 H), 1.95 (s, 3 H), 0.90 (m, 6 H); IR 2100 (isothiocyanate), 1680 (C=O), and 1650 cm⁻¹ (C=S).

8-[4-[[[[[2-[[[5-[[[2-(Dimethylamino)ethyl]amino]-(thiocarbonyl)]amino]-3-isothiocyanatophenyl](thiocarbonyl)]-amino]ethyl]amino]carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine(5f)—mp >250 °C; NMR (DMSO-*d*₆) δ 8.35 (m, 1 H), 8.10 (d, 2 H, *J* = 8.7 Hz), 7.60-7.20(m, 3 H), 7.10 (d, 2 H, *J* = 8.7 Hz), 4.55 (s, 2 H), 4.05 and 3.90 (each m, 2 H), 3.65 (m, 2 H), 2.60 (m, 2 H), 2.30 (s, 6 H), 1.75 and 1.60 (each m, 2 H), 0.9 (m, 6 H) ppm.

8-[4-[[[[[2-[[[5-[[[2-(Acetylamino)ethyl]amino]-(thiocarbonyl)]amino]-3-isothiocyanatophenyl](thiocarbonyl)]-amino]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (5g)—mp >250 °C; NMR (DMSO-*d*₆) δ 8.35 (m, 1 H), 8.09 (d, 2 H, *J* = 8.7 Hz), 7.55-7.25 (m, 3 H), 7.10 (d, 2 H, *J* = 8.7 Hz), 4.60 (s, 2 H), 4.05 and 3.90 (each m, 2 H), 3.50 (m, 4 H), 1.80 and 1.62 (each m, 2 H), 0.9 (m, 6 H) ppm.

8-[4-[[[[[2-[[[5-[[[2-[[3-(4-Hydroxyphenyl)propionyl]amino]ethyl]amino]carbonyl]-3-isothiocyanatophenyl](thiocarbonyl)]-amino]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (5m)—XAC (24 mg, 56 μmol) and 3-[2-[[[2-[(3,5-diisothiocyanatobenzoyl)amino]ethyl]amino]carbonyl]ethyl]phenol (35 mg, 82 μmol) were suspended in 1 mL of dimethylformamide and sonicated until a solution formed. After 1 h, ether was added, and a precipitate formed. The product (**5m**) was recrystallized from dimethylformamide/ether to give 39.9 mg (83% yield): NMR (DMSO-*d*₆) δ 9.93 (s, 1 H, CSNHAr), 9.12 (s, 1 H, ArOH), 8.36 (t, 1 H, NH of oxymethylamide), 8.55 and 7.93 (each t, 1 H, NH), 8.07 (d, 2 H, *J* = 8.6 Hz, 8-phenyl ring, meta to ether), 7.75 (m, 1 H, NH), 7.95, 7.77, and 7.58 (each s, 1 H, 2,4,6-aryl protons), 7.10 (d, 2 H, *J* = 8.6 Hz, 8-phenyl ring, ortho to ether), 6.96 (d, 2 H, *J* = 8.3 Hz, meta to phenol), 6.63 (d, 2 H, *J* = 8.3 Hz, ortho to phenol), 4.58 (s, 2 H, CH₂OAr), 4.01 and 3.87 (each m, 2 H, C α of Pr), 3.62 (br m, 2 H, CH₂NHCS), 3.2-3.4 (m, 6 H, CH₂NH), 2.68 (t, 2 H, CH₂ α to phenol ring), 2.29 (t, 2 H, CH₂ β to phenol ring), 1.75 and 1.60 (each m, 2 H, C β of Pr), 0.9 (m, 6 H, C γ or Pr) PPM.

8-[4-[[[[[2-[[[5-[[[4-[[4-(Fluoromethyl)benzoyl]-amino]butyl]amino](thiocarbonyl)]-amino]-3-isothio-cyanatophenyl](thiocarbonyl)]-amino]ethyl]amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (5r)—Compound **22** (100 mg, 0.15 mmol) and 4-[[4-(fluoromethyl)benzoyl]amino]butylamine trifluoroacetate (31.4 mg, 0.10 mmol) were added

to 0.5 mL of dimethylformamide with stirring, followed by the addition of 14 μ L of triethylamine. After stirring overnight at room temperature, the mixture was applied to a semipreparative thin-layer chromatography plate (silica, 1000 microns, Analtech, Newark, DE) and chromatographed with a mixture of chloroform/methanol/acetic acid (90/10/1, by volume). A major band was identified as homogeneous product by TLC, NMR, and IR: yield 20 mg (22%). A californium plasma desorption spectra (positive ions) showed peaks at m/z 956.8 ($M + H + 2 Na$), 924.8 ($M + H + Na$), 505.1, 447.2, and 389.2.

8-[4-[[[2-[[3,5-Diisothiocyanatophenyl](thiocarbonyl)]amino]ethyl]amino]carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine (22)—was synthesized by the above method from compound **21**: mp >250 °C; NMR (DMSO- d_6) δ 8.30 (m, 1 H), 8.05 (d, 2 H, $J = 8.5$ Hz), 7.46 (s, 2 H), 7.24 (s, 1 H), 7.09 (d, 2 H, $J = 8.5$ Hz), 4.58 (s, 2 H), 4.01 (m, 2 H), 3.87 (m, 2 H), 1.74 (m, 2 H), 1.58 (m, 2 H), 0.90 (m, 6 H) ppm. Accurate mass (FAB) consistent with the assigned structure.

3,5-Bis[(*tert*-butyloxycarbonyl)amino]benzoic Acid (6)

3,5-Diaminobenzoic acid dihydrochloride (10 g, 44 mmol) was added to 120 mL of 1 M aqueous sodium hydroxide. Methanol (60 mL) was added. Di-*tert*-butyl dicarbonate (19 g, 87 mmol) dissolved in 15 mL of methanol was added to the diamine with stirring. After 12 h, the reaction was acidified with citric acid and extracted with ethyl acetate. The organic layer was dried (Na_2SO_4) and the solvent evaporated. Addition of ether to the residue resulted in the precipitation of salts, which were removed by filtration. The ether solution was extracted with cold 1 N HCl. After separation, the organic solvent was evaporated in vacuo, leaving a white solid: yield 9.65 g (62%); mp 113–116 °C.

Methyl 3,5-Bis[(*tert*-butyloxycarbonyl)amino]benzoate (7)

3,5-Bis[(*tert*-butyloxycarbonyl)amino]benzoic acid (5.00 g, 14.2 mmol) was esterified in methanol using EDAC6 (14.2 mmol) and 4-(dimethylamino)pyridine (2.4 mmol). After 2 h the product precipitated as a solid and was collected, washed with a small amount of methanol, and dried in vacuo. A second crop was collected, yielding a total of 3.64 g (70%) of product.

2-[[3,5-Bis[(*tert*-butyloxycarbonyl)amino]benzoyl]-amino]ethylamine (8)

Methyl 3,5-bis[(*tert*-butyloxycarbonyl) amino]benzoate (1.98 g, 5.4 mmol) was dissolved in a minimum of ethylenediamine and allowed to react at room temperature for 3 days. Water was added, and the product (1.84 g, 86% yield) precipitated as a white solid.

N-Succinimidyl 3,5-Bis[(*tert*-butyloxycarbonyl)-amino]benzoate (9)

3,5-Bis[(*tert*-butyloxycarbonyl)-amino]benzoic acid (350 mg, 1 mmol) and 230 mg (1.2 equiv) of EDAC were placed in 30 mL of CH_3CN , and the solution was stirred at room temperature for 1 h. To this was added 130 mg (1.2 equiv) of *N*-hydroxysuccinimide, and the mixture was stirred overnight. The mixture was diluted with 150 mL of ethyl acetate and washed with 100 mL of pH 4 NaH_2PO_4 (0.5 M). The solvent was removed on a rotary evaporator, and the residual solid was placed on a Chromatotron and eluted with ether/petroleum ether (1/1). The fractions containing the first band eluted were combined and evaporated to dryness to provide 301 mg (67%) of product: NMR (DMSO- d_6) δ 9.75 (s, 2 H), 7.98 (s, 1 H), 7.90 (s, 2 H), 3.35 (s, 4 H), 1.45 (s, 18 H) ppm.

⁶¹1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

4-[[[2-[[3,5-Bis[(*tert*-butyloxycarbonyl)amino]-benzoyl]amino]ethyl]amino]carbonyl]ethyl]phenol (10b)—2-[[3,5-Bis[(*tert*-butyloxycarbonyl)amino]benzoyl]-amino]ethylamine (8, 185 mg, 0.47 mmol) and *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (Fluka, 105 mg, 0.40 mmol) were dissolved with sonication in 2 mL of MeOH/dimethylformamide (1/1). After 1 h, 3 mL of water was added, and an oil separated. The resulting residue was purified by precipitation from ethyl acetate/hexanes resulting in 136 mg of product [63% yield; homogeneous by TLC (R_f = 0.60, silica, chloroform/methanol/acetic acid 85/10/5)].

4-[[2-[[3,5-Bis[(*tert*-butyloxycarbonyl)amino]benzoyl]amino]ethyl]amino]-7-nitrobenzofurazane (10g)—Compound 8 (462 mg, 1.0 mmol) and 4-chloro-7-nitrobenzofurazane (NBD-Cl, 0.25 g, 1.25 mmol) were added to 50 mL of acetonitrile and stirred overnight. The resulting dark solution was reduced in volume by evaporation and filtered through silica gel to remove polar impurities. The filtrate was purified using a Chromatotron, eluting with ether to yield 0.34 g of the product as an orange solid (60% yield).

5-[[[2-[[3,5-Bis[(*tert*-butyloxycarbonyl)amino]-benzoyl]amino]ethyl]amino](thiocarbonyl)]-amino]fluorescein (10h)—Compound 8 (0.23 g, 0.5 mmol) and fluorescein isothiocyanate isomer 1 (0.23 g, 0.6 mmol) were added to a small vial followed by 1 mL of DMF, and the mixture was sonicated for 5 min. The resulting viscous solution was applied directly to four 20 × 20 cm preparative silica TLC plates (1000 μm) and chromatographed with ethyl acetate containing 1 % acetic acid. The major band (R_f ≈ 0.6) was removed and eluted with methanol. The extract was evaporated and crystallized from a mixture of methanol/ether/petroleum ether, leaving 0.20 g (51 % yield) of **10h** as an orange solid. Fast atom bombardment (positive) mass spectroscopy showed peaks at m/z 806 ($M + 1 + Na$), 784 ($M + 1$), 622, 390, 223, 154.

[[[2-(Dimethylamino)ethyl]amino]carbonyl]-3,5-dinitrobenzene (12b)—*N,N*-Dimethylethylenediamine (2.2 mL) and triethylamine (5 mL) were dissolved in 100 mL of DME. A solution of dinitrobenzoyl chloride (4.6 g, 20 mmol) in DME (25 mL) was added, and the mixture was stirred overnight. Sodium hydroxide (10%, 150 mL) was added and the mixture was extracted with a 1/1 mixture of ether/ethyl acetate. The combined organic extracts were evaporated to dryness, leaving 2.5 g (44%) yield of the product, an oil. A final purification was carried out with a Chromatotron, eluting with ethyl acetate/methanol (1/10). The product was homogeneous by TLC (R_f = 0.69, silica, chloroform/methanol/ NH_4OH 60/40/2); NMR ($CDCl_3$) δ 9.15 (s, 1 H), 9.04 (s, 2 H), 3.60 (m, 2 H), 2.31 (s, 6 H) ppm; IR 1650 cm^{-1} (amide).

[[[2-(Trimethylammonio)ethyl]amino]carbonyl]-3,5-dinitrobenzene iodide (12c)—Compound **12b** (0.50 g, 1.77 mmol), methyl iodide (1.0 mL), and diisopropyl-ethylamine (0.5 mL) were added to methanol (50 mL) and stirred overnight at room temperature. The resulting precipitate was collected by filtration and washed with acetone. The product (725 mg) was dried in vacuo.

4-[2-[[[2-[(3,5-Diaminobenzoyl)amino]ethyl]amino]-carbonyl]ethyl]phenol (13e)—4-[2-[[[2-[[3,5-Bis[(*tert*-butyloxycarbonyl) amino]benzoyl]amino]ethyl]amino]carbonyl]ethyl]phenol (**10b**) was deprotected in 95% yield upon dissolving in neat trifluoroacetic acid for 5 min, followed by evaporation and precipitation with ether. R_f = 0.10 (silica, chloroform/methanol/acetic acid 85/10/5).

⁷1,2-Dimethoxyethane.

General Methods for the Preparation of Diisothio-cyanates (Compounds 14, 19, 26, 28, 33)**Method A (Used for Ethyl 3,5-Diisothiocyanatobenzoate (26), 3,5-Diisothiocyanatobenzyl Alcohol (28), and 2,6-Diisothiocyanatopyridine (33))—**

The diisothiocyanates prepared in this section were derived from the commercially available aromatic diamines. To a solution of 10 mM of the diamine dissolved in 50 mL of a 1/1 mixture of EtOH/CH₃CN, containing 4 g (excess) of NaHCO₃, cooled to 0 °C in an ice/MeOH bath, was added 1.5 mL (excess) of thiophosgene all at once via syringe. This was allowed to warm to room temperature over a 1-h period, then quenched by the cautious addition of 100 mL of pH 4 NaH₂PO₄. The mixture was extracted three times with 100-mL portions of ethyl acetate. The extracts were combined, dried over anhydrous Na₂SO₄, and filtered, and solvent was removed by evaporation. Final purification was effected on a Chromatotron using ethyl acetate/petroleum ether (1/4) as eluent: NMR (**26**, CDCl₃) δ 7.76 (s, 2 H), 7.20 (s, 1 H), 4.40 (q, 2 H), 1.40 (t, 3 H) ppm; (**28**, CDCl₃) δ 7.14 (s, 2 H), 6.95 (s, 1 H), 4.69 (s, 1 H) ppm.

Method B (Used for 3,5-Diisothiocyanatobenzamide (14a))—The following procedure was employed in those cases where the necessary aromatic diamine was prepared from its dinitro precursor. The diamines were not isolated but were used *in situ* to prepare the desired diisothiocyanate. A solution of 10 mmol of the appropriate, commercially available, dinitro compound in 50 mL of MeOH was treated with 100 mg of 10% Pd on carbon catalyst. The mixture was hydrogenated in a Parr hydrogenator and shaken at room temperature under 25 psi of H₂ for 2 h. TLC was used to determine reaction completion. When finished, the solution was filtered through a plug of Celite to remove the catalyst, and the methanolic solution of diamine was treated directly to the conditions of method A (presuming complete reduction) to prepare the desired diisothiocyanate. Final purification was effected on a Chromatotron using ethyl acetate/petroleum ether (1/4) as eluent: NMR (DMSO-*d*₆) δ 8.15 (s, 1 H), 7.86 (s, 2 H), 3.35 (s, 2 H) ppm.

Method C (Used for *N*-[2-(Acetylamino)ethyl]-3,5-diisothiocyanatobenzamide (14b), *N*-[2-(Dimethyl-amino)ethyl]-3,5-diisothiocyanatobenzamide (14d)]—To a stirring solution of 20 mM of either *N*-acetyylethyl-enediamine (for **14b**) or *N,N*-dimethylethylenediamine (for **14d**) and excess triethylamine contained in 100 mL of DME at room temperature was slowly added 20 mM of 3,5-dinitrobenzoyl chloride in 20 mL of DME. A thick precipitate formed, and the mixture was allowed to stir overnight, whereupon 150 mL of 10% aqueous NaOH was added. The resulting mixture was extracted with 2 × 300 mL portions of ethyl acetate, and the combined extracts were washed sequentially with 0.5 N HCl and half-saturated NaCl solution. The organic fraction was separated and dried over anhydrous Na₂SO₄, then the solvent was removed by evaporation. The unpurified dinitro amide was then subjected to the conditions of method B to yield the corresponding diamine, then to method A to ultimately yield the listed diisothiocyanates. Final purification was either on a Chromatotron using ethyl acetate/petroleum ether (1/4) or by recrystallization from ethyl acetate: NMR (**14b**, DMSO-*d*₆) δ 7.85 (m, 2 H), 7.55 (m, 1 H), 4.2-4.0 (m, 4 H), 1.90 (s, 3 H) ppm; (**14d**, DMSO-*d*₆) δ 9.10 (m, 1 H), 7.92 (s, 2 H), 7.78 (s, 1 H), 3.65 and 3.28 (each m, 2 H), 2.80 (s, 6 H) ppm.

Method D (Used for *N*-[3,5-Diisothiocyanatophenyl]-*N'*-[2-(acetylamino)ethyl]thiourea (19a), *N*-[3,5-Diisothiocyanatophenyl]-*N'*-[2-(dimethylamino)-ethyl]thiourea (19b)]—To 500 mg (2 mmol) of triisothiocyanatobenzene (**21**) contained in 50 mL of DME, cooled to 0 °C in a bath of ice/MeOH, was added 0.66 mmol of either *N*-acetyylethylenediamine (for **19a**) or *N,N*-dimethylethylenediamine (for **19b**). This was stirred in the cold for 1 h, and the solvent was removed by evaporation. The remaining residue was placed on a Chromatotron with the

initial eluent being ethyl acetate/petroleum ether (1/4) until the first band (triisothiocyanatobenzene) was removed, then replaced with pure ethyl acetate until the second band (the desired homogeneous product) was collected: NMR (**19a**, CDCl₃) δ 7.35 (m, 2 H), 6.86 (s, 1 H), 6.75 (m, 1 H), 3.48 (m, 4 H), 2.05 (s, 3 H) ppm; (**19b**, CDCl₃) δ 7.20 (s, 2 H), 6.80 (s, 1 H), 3.65 (m, 2 H), 2.65 (m, 2 H), 2.35 (s, 3 H) ppm; CIMS (**19b**) m/z 338 (M + 1), 304, 293, 208; (**19b**) R_f = 0.42 (silica, ethyl acetate/methanol/triethylamine 75/25/1).

Method E (Used for Converting 13 to 14 or 18 to 19)

4-[2-[[[2-[[3,5-Diisothiocyanatobenzoyl]amino]-ethyl]amino]carbonyl]ethyl]phenol (14e): 3-[2-[[[2-[(3,5-Diaminobenzoyl)amino]ethyl]amino]carbonyl]ethyl]-phenol (80 mg, 234 μ mol) was dissolved in a mixture of chloroform (1 mL), dimethylformamide (0.5 mL), and saturated sodium bicarbonate (1 mL). With stirring, thiophosgene (60 μ L, 0.8 mmol) was added. After 1 h, the organic layer was washed with water treated with ether. A white precipitate was collected: yield 56 mg (56% yield).

4-[2-[[[3,5-Diisothiocyanatobenzoyl]amino]ethyl]-amino]-7-nitrobenzofurazane (14j): Compound **10g** (134 mg, 0.24 mmol) was treated with 0.5 mL of trifluoroacetic acid for 10 min at room temperature. The trifluoroacetic acid was removed under a stream of nitrogen, and the residue was dissolved in 5 mL of an equivolume mixture of methanol/acetonitrile. Sodium bicarbonate (0.2 g) was added to the stirred solution, followed by 100 μ L of thiophosgene. After 10 min, 5 mL of aqueous 0.5 M monobasic sodium phosphate was added. The mixture was extracted twice with 15 mL of ethyl acetate. The combined organic extracts were dried in vacuo to yield the product, which was homogeneous by TLC (R_f = 0.57, silica, eluting with ether).

1,3,5-Triisothiocyanatobenzene (21)

3,5-Dinitroaniline, in methanol solution, was reduced to 1,3,5-triaminobenzene (**20**) in quantitative yield with hydrogen gas (20 psi) and 10% Pd/C at room temperature in a Parr shaker.

1,3,5-Triaminobenzene (**20**; 0.50 g, 4.06 mmol) and sodium bicarbonate (3 g) were added to 50 mL of a 1/1 (v/v) mixture of methanol and acetonitrile, and the mixture was cooled to -5 °C in an ice/methanol bath. Thiophosgene (2 mL) was added in one aliquot with vigorous stirring. The reaction was allowed to warm to room temperature slowly. A 0.5 M solution of sodium phosphate monobasic (100 mL) was added. The mixture was extracted three times with ether. The organic phase was evaporated to dryness, leaving a solid residue which was purified with a Chromatotron, eluting with 10% ethyl acetate in petroleum ether: 84% yield; NMR (CDCl₃) δ 7.01 (s) ppm; IR 2100 cm⁻¹ (isothiocyanate); EIMS m/z (M⁺) 249; mp 64–66 °C (lit. (16) mp 63–65 °C).

3-[(*tert*-Butyloxycarbonyl)amino]anilin(29)

1,3-Phenylenediamine (1.41 g, 13 mmol) and di-*tert*-butyl dicarbonate (2.73 g, 13 mmol) were dissolved in methanol (30 mL). After 1 h, ethyl acetate was added, and the mixture was extracted twice with water. The organic layer was dried by evaporation, and the product was purified on a Chromatotron to give 1.0 g (37 %) of the product as a yellow oil, which crystallized slowly: mp 109–110 °C. Anal. (C₁₁H₁₆N₂O₂) C, H, N.

3-[(*tert*-Butyloxycarbonyl)amino]-1-isothiocyanatobenzene (30)

Compound **29** (0.51 g, 2.45 mmol) was combined with a rapidly stirred mixture of chloroform (60 mL), water (20 mL), and sodium bicarbonate (0.50 g), and the mixture was treated with thiophosgene (0.25 mL) added rapidly. After 10 min the layers were separated,

and the lower layer was dried in vacuo. The residue was redissolved in ethyl acetate and treated with petroleum ether, and a solid impurity was removed by filtration. The filtrate was dried in vacuo, leaving the pure product as a solid: 0.47 g; 77% yield; mp 82–83 °C. Anal. (C₁₂H₁₄N₂O₂S₂) C, H, N, S.

8-[4-[[[[[2-[[[3-Aminophenyl]amino](thiocarbonyl)]amino]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (34)—was obtained in quantitative yield upon treatment of compound **35** with trifluoroacetic acid for 10 min. The product was homogeneous by TLC (*R_f* = 0.76, silica, chloroform/methanol/acetic acid 85/10/5).

8-[4-[[[[[2-[[[3-[(*tert*-Butyloxycarbonyl)amino]-phenyl]amino](thiocarbonyl)]amino]ethyl]amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (35)—Compound **1** (50 mg, 117 μmol) and compound **30** (40 mg, 0.16 mmol) were added to 1 mL of DMF⁸ and stirred for 1 h. Ethyl acetate (30 mL) and ethylenediamine (0.1 mL, scavenger for unreacted isothiocyanate) were added, and the solution was extracted with water, sodium monophosphate, and saturated sodium chloride. The organic phase was dried (Na₂SO₄), the solvent evaporated, and the residue recrystallized from DMF/ethyl acetate/petroleum ether to provide 44 mg (56%) of pure product.

1,3-Dipropyl-8-[4-[[[[[2-[[[3-[(*N*-succinimidyl)oxy]-carbonyl]phenyl]amino](thiocarbonyl)]amino]ethyl]-amino]carbonyl]methyl]oxy]phenyl]xanthine (37)—Compound **1** reacted with 3-isothiocyanatobenzoic acid (mp 161–162 °C) in dimethylformamide to provide 8-[4-[[[[[2-[[[3-(3-carboxyphenyl)amino](thiocarbonyl)]amino]ethyl] amino] carbonyl] methyl]oxy]phenyl]-1,3-dipropylxanthine (**36**).

Compound **36** (61 mg, 0.11 mmol), *N*-hydroxysuccinimide (40 mg), and EDAC (66 mg) were mixed in 2 mL of dimethylformamide. A solution formed and was allowed to react overnight. Two volumes of water were added, and the precipitate was filtered to yield the product (50 mg).

8-[4-[[[[[2-[[[6-Isothiocyanatopyrid-2-yl]amino](thiocarbonyl)]amino]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (40)—2,6-Diaminopyridine was converted in 41% yield to 2,6-diisothiocyanatopyridine **33** with thiophosgene and sodium bicarbonate (see procedure for **21**) in an equivolume mixture of acetonitrile/ethanol; mp 48–49 °C; (DMSO-*d*₆) δ 8.00 (t, 3 H), 7.30 (d, 1 H) ppm; IR 2100 cm⁻¹. Anal. (C₇H₃N₃S₂) C, H, N, S.

Compound **1** (25 mg, 58 μmol) and compound **33** (44 mg, 0.23 mmol) were added to 0.5 mL of DMF and sonicated for 5 min. Ether (3 mL) was added; the precipitate was collected by centrifugation, washed with ethyl acetate, and dried in vacuo to yield 26 mg (72 %) of product **40**: mp >250 °C; NMR (DMSO-*d*₆) δ 8.38 (m, 1 H), 8.05 (d, 2 H, *J* = 8.8 Hz), 7.83 (t, 1 H, *J* = 8.0 Hz), 7.15 (d, 1 H, *J* = 8.0 Hz), 7.06 (d, 2 H, *J* = 8.8 Hz), 6.98 (d, 1 H, *J* = 8.0 Hz), 4.57 (s, 2 H), 4.02 and 3.87 (each m, 2 H), 1.74 and 1.58 (each m, 2 H), 0.9 (m, 6 H) ppm. Anal. (C₂₈H₃₁N₉O₄S₂·0.5H₂O) C, H, N, S.

Solubility of Analogues

25 μL of a DMSO solution containing ca. 10 mM of the xanthine to be assayed was mixed thoroughly with 675 μL of pH 7.2 phosphate buffer. This mixture was vortexed for 1 min

⁸Dimethylformamide.

and then left at room temperature for 2–3 h. In each case (except where noted) a fine precipitate resulted and was removed by centrifugation at the end of the waiting period. A 100- μ L aliquot of the supernatant then was added to 1.0 mL of methanol, and the xanthine concentration was measured spectrophotometrically using an extinction coefficient of 28 180 at 310 nm for XAC and its N-acylated derivatives. Each xanthine was assayed three times, and the concentrations reported in Table IV represent the averages of three determinations.

Competitive Binding Assay in Rat Brain Using [3 H]-PIA and [3 H]CGS21680

Inhibition of binding of 1 nM *R*-PIA⁹ (specific activity 42.5 Ci/mmol) to A₁ adenosine receptors in rat cerebral cortex membranes was assayed as described.¹³ Stock solutions of the xanthine derivatives in DMSO (1–10 mM) were prepared. Inhibition of binding by a range of concentrations of a xanthine derivative was assessed in triplicate in at least three separate experiments. Separation of bound and free radioligand was accomplished by rapid filtration using a Brandel cell harvester (Brandel, Gaithersburg, MD) and a Whatman GF/B glass-fiber filter. Nonspecific binding was determined in the presence of 10 μ M 2-chloroadenosine. At least seven different concentrations spanning 4 orders of magnitude, adjusted appropriately for the IC₅₀ of each compound, were used. IC₅₀ values, computer-generated with a non-linear regression formula of the GraphPAD program (Institute for Scientific Information), were converted to apparent K_i values with a K_D value for [3 H]PIA of 1.0 nM (13) and the Cheng–Prusoff equation (17). The use of K_i is justified for most of the compounds, since the degree of irreversible binding at A₁ receptors is relatively small in the range of the IC₅₀ concentration (except for compounds **5c** and **22**). To account for the few compounds in which the K_i is expected to be shifted significantly due to partial irreversible inhibition of A₁ receptors in the pertinent concentration range, the expression “apparent K_i ” value has been used.

Affinity at rat striatal A₂ receptors was measured similarly using [3 H]CGS21680¹⁰(18). IC₅₀ and K_i values were determined as described above with a K_D value for [3 H]CGS21680 of 15 nM.

Irreversible Binding to Bovine Brain

For studies of irreversible incorporation, membranes from bovine brain were prepared as described (10) and then incubated with the indicated (Table IV) concentration of ligand for 45 min at 37 °C. Membranes were then washed three times by sequential resuspension and centrifugations with buffer A (50 mM Tris-HCl, 10 mM magnesium chloride, and 1 mM ethylenediamine tetraacetic acid, disodium salt) containing 0.02% Chaps.¹¹ Membranes were then suspended in buffer A containing 0.1 mM IBMX¹² and incubated at 25 °C for 18 h with shaking.¹³ Membranes were then washed twice with buffer A, treated with adenosine deaminase (3 units/mL), and used in radioligand saturation studies as described (10) with a range of concentrations of [125 I]APNEA.¹⁴ For xanthine derivatives that irreversibly inhibited receptor binding, such as m-DITC-XAC, initial experiments demonstrated that complete incorporation had occurred by 30 min (data not shown).

⁹(*R*)-*N*⁶-(2-Phenylisopropyl)adenosine.

¹³The long treatment with IBMX was found necessary to remove all of the nonincorporated ligands from membranes (10). Multiple washes (up to eight) with buffer alone were insufficient to remove all the reversibly bound ligands (10).

¹⁰2-[[2-[4-(2-Carboxyethyl)phenyl]ethyl]amino]adenosine-5'-*N*-ethylcarboxamide.

¹¹3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate.

¹²3-Isobutyl-1-methylxanthine.

¹⁴[125 I]-*N*⁶-[2-(4-Amino-3-iodophenyl)ethyl]adenosine.

RESULTS

Chemistry

The 1,3-diisothiocyanate structure was preserved in the synthetic design of cross-linking reagents **4** due to its proven merit both in reacting with an amine-containing xanthine pharmacophore (**1**) and in acylating receptor residues (10). The symmetry of compound **4** permits multiple synthetic pathways to a desired ligand, and several routes were investigated for feasibility during this study. The pathways may be conveniently broken into two schemes leading to cross-linker **4** and its subsequent transformation to xanthine conjugate **5**, depending on whether group R' of Figure 1 is linked to the benzene ring in the 5-position through an amide (Figure 2) or a thiourea group (Figure 3). As shown in Figure 3, a central intermediate in the synthesis of 1,3,5-thiourea derivatives is symmetrical 1,3,5-triisothiocyanatobenzene (**21**). Alternately, hydroxymethyl, ester, and carboxylic anhydride groups at the 5-position (Figure 4) were explored. The synthesis and characterization of the intermediates are summarized in Table I.

The substituent R' is intended for enhancing solubility, for introducing chemical or photochemical reactivity (19), or as a spectroscopic marker for characterization of the receptor (13). This reporter group may be introduced by two pathways: either (1) by incorporation in a symmetrical diisothiocyanate intermediate designed to react with **1** (e.g. **14** or **19**) or (2) by addition to a reactive center (an isothiocyanate) already linked to the pharmacophore (as in **22**). Monosubstituted conjugates of **14** or **19** (general structure **5**) were isolated following treatment of **1** with an excess of the necessary diisothiocyanate in dimethylformamide, using sonication to effect gradual solubilization of **1**. The product then precipitated upon addition of either diethyl ether or ethyl acetate. The second pathway required a roughly 2-fold molar excess of compound **21** to form **19**. Both approaches were explored in the development of the trifunctional methodology and are presented in this paper. Table I lists representative xanthines synthesized (structures in Table II). Compounds **5k**, **5l**, **5o**, **5p**, and **5r** were prepared via compound **22**.

The first retrosynthetic operation on general structure **4** reveals a diamine (compounds **13** and **18**, in Figures 2 and 3, respectively). This generalized diamine was prepared by different routes, depending on the nature of the third substituent, R'. The aryl 1,3-diamines were obtained either from the corresponding 1,3-bis[*tert*-butyloxycarbonyl]amino compound (**10**) by acid deprotection or through reduction of the 1,3-dinitro precursors (**12** or **17**), which were derived from 3,5-dinitrobenzoyl chloride (**11**) or were commercially available. Structure **12** was readily reduced by catalytic hydrogenation using 10% Pd/C; however, the same procedure failed to reduce the nitro groups of the analogous thiourea **17**. The reduction of the cationic 2-(trimethylamino)ethyl carboxamide dinitro intermediate **12c**, desired for purposes of water solubility, was sluggish. An alternative method, used successfully for compound **17** (R'' = (CH₂)₂NHCOCH₃), was reduction using zinc in acetic acid (20). Diamino carboxamides **13** were usually not isolated, but were subsequently converted to the corresponding diisothiocyanates **14**. The 1,3-diisothiocyanate intermediates included in this study (**14** and **19**) were prepared either by treatment of their penultimate 1,3-diamines with thiophosgene in the presence of base (10) or by addition of a primary amine to 1,3,5-triisothiocyanatobenzene (**21**, isothiocyanic acid, *s*-phenenyl triester) (16). The 5-thiourea-substituted cross-linkers (Figure 3) were prepared principally from **21**. Thiourea diisothiocyanates **19** were prepared by the reaction of excess **21** with amines such as *N,N*-dimethylethylenediamine or *N*-acetythylenediamine in dimethoxyethane as presented in Figure 3.

As shown in Figure 2, a convenient means of introducing a variety of reporter groups was via an aliphatic amine-functionalized precursor, **8**. The two amino groups of 3,5-diaminobenzoic acid were protected as *tert*-butyl car. bamates (Boc) (**10**), and resulting acid **6** was esterified in methanol with EDAC and 4-(dimethylamino)pyridine. The ester was aminolyzed in neat ethylenediamine to give **8**. This intermediate reacted either with an isothiocyanate (such as fluorescein isothiocyanate) (**13**) to afford an N-substituted thiourea or with an activated acyl group (such as *N*-succinimidyl 3-(4-hydroxyphenyl)propionate) (**21**) to give a carboxamide. This was followed by removal of the Boc protecting groups and treatment with thiophosgene. Alternatively, 3,5-bis[(*tert*-butyloxycarbonyl)-amino]benzoic acid (**6**) may be converted to its hydroxysuccinimide active ester (**9**), which may be conveniently coupled to an amine-bearing probe (such as 5-[(2-aminoethyl)thioureidyl]fluorescein) (**22**) then deprotected and transformed into the corresponding diisothiocyanate in the aforementioned manner (Figure 2).

We attempted to prepare analogues in which group R' was anionic, for increasing aqueous solubility. Treatment of 3,5-diaminobenzoic acid with the standard conditions (CSCl₂, base) failed to afford the expected 3,5-diisothiocyanatobenzoic acid. Instead, the isolated product (Figure 4) was the tetraisothiocyanato benzoic anhydride **24**, resulting from dehydration of the acid. Changes in reaction conditions (solvent, base, or order of addition) had no effect on the reaction outcome. The anhydride was used, however, in preparing a conjugate with xanthine **1**, and reaction occurred only at the isothiocyanate group. Another route to an acidic analogue involved the reaction of 2-aminoethanesulfonic acid with 3,5-dinitrobenzoyl chloride (**11**), followed by catalytic reduction of the nitro groups to amines, then conversion to the diamine to the diisothiocyanate. The isolated diisothiocyanate sulfonic acid, however, failed to couple with **1**. A third route involved reacting glycine *tert*-butyl ester with excess triisothiocyanatobenzene (**21**) to yield thiourea-linked diisothiocyanate **19**. Reaction of this intermediate with **1** afforded desired product **5h**, but attempted cleavage of the *tert*-butyl ester group by brief treatment with CF₃CO₂H, under which conditions the aryl isothiocyanate is preserved, resulted in loss of the glycine side chain.

As shown in Figure 4, diamino ester **25** was also used to prepare ethyl 3,5-diisothiocyanatobenzoate (**26**) and 3,5-diaminobenzyl alcohol (**27**) by reduction of the ester. Compound **27** was converted to diisothiocyanate **28** by the usual thiophosgene procedure.

Table I lists the xanthine analogues synthesized, including monosubstituted model compounds (**34–40**) and derivatives containing both an isothiocyanate group and chemically reactive (**5i** and **5j**), photoreactive (**5k** and **5l**), spin-labeled (**5o**), fluorescent (**5p** and **5q**), fluorine-containing (**5r**, as a model for radiofluorination) (**15**), or iodinated (**5l** and **5m**) (**21**) reporter groups or solubilizing (**5a–5h**) groups.

The types of groups chosen for enhancing solubility consisted of acidic, basic, ionic (quaternary amine), and hydrophilic (i.e. alcohol, amide and ester) moieties. The kinds of linking units explored in the derivatives were carboxamide, thiourea, and methylene. Pyridine isothiocyanate **40** was included in hopes that the annular heteroatom would afford some degree of solubilization or altered receptor subtype selectivity.

Biology

The apparent K_i values (nanomolar) for A₁ and A₂ binding in rat brain are presented in Table II. The inhibition of binding of [³H]-N⁶-(2-phenylisopropyl)adenosine at A₁ receptors in the cortex (**13**) and the inhibition of binding of [³H]CGS21680 at A₂ receptors in the striatum (**18**) were measured. The relative selectivities are expressed as the ratio of K_i values. The most potent and selective of the potentially irreversibly binding xanthines were **5d**, **5e**, **5h**, **5m**, and **22**.

Several model compounds (**34–40**) having substitutions in the meta position of the thiourea-linked ring were prepared to probe the feasibility of chain extension from this site. The 3-amino (**34**) and 3-[(*tert*-butyloxycarbonyl)-amino] (**35**) derivatives were nearly equipotent in binding to A₁ receptors, indicating a tolerance for sterically large groups in this region. A carboxylic derivative (**36**) was 16-fold less potent than the corresponding aniline (**34**), indicating a detrimental effect of anionic groups in receptor binding, as has been previously noted for other 8-substituted xanthines (5). When blocked as a succinimido ester (**37**), which itself is potentially reactive toward amine nucleophiles on the receptor, the high potency was restored. Two long-chain derivatives (**38** and **39**) were of intermediate potency at A₁ receptors, suggesting that chain extension at the meta position is tolerated in receptor binding. A 2,6-disubstituted pyridine derivative (**40**) was 7-fold less potent than the benzene equivalent (**2**) at A₁ receptors, yet nearly equipotent at A₂ receptors.

Among trisubstituted isothiocyanate xanthine derivatives **5a-r** and **22** there was a large variation in potencies at both A₁ and A₂ receptors. Diisothiocyanate xanthine **22** was nearly as potent as **2** at A₁ receptors, yet 6-fold more selective. Among simple 5-substitutions, a carboxamide (**5c**) tended toward higher potency, suggesting that this linkage would be tolerated in extended structures, as was found for ethylenediamine derivatives **5d** and **5e**. The amide linkage was found to be preferred over the thiourea linkage in receptor binding, as in the corresponding compounds **5f** and **5g**. In comparison to ethylenediamine derivative **5f**, a glycine *tert*-butyl ester derivative (**5h**) displayed enhanced affinity for A₁ receptors and 480-fold selectivity.

The incorporation of chemically (**5i** and **5j**) and photochemically (**5k** and **5l**) reactive groups in the 5-position did not prevent receptor binding. (*p*-Hydroxyphenyl)-propionyl derivative **5m** and 4-azidosalicylic acid derivative **5l** are potentially iodlatable, for the covalent introduction of a radiotracer into the affinity-labeled receptor. Compound **5l** moreover could conceivably bridge two sites on the receptor molecule covalently, after photolysis of the azido group. Compound **5m** is of higher affinity for A₁ receptors and is 200-fold selective. A biotinylated probe (**5n**), potentially useful for receptor isolation and visualization (23), was 530-fold selective for A₁ receptors. Of the fluorescent-labeled ligands (**5p** and **5q**), an NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl)derivative (amide linked in the 5-position) displayed intermediate affinity for A₁ receptors and 60-fold selectivity.

The degree of irreversible binding was determined as previously described (10) in bovine brain. Saturation curves for [¹²⁵I]APNEA binding to A₁ adenosine receptors in bovine brain membranes, before and after 45-min incubation with the isothiocyanate-bearing xanthine, were measured (Figure 5). The percent inhibition (reduction in $B_{\max}$ relative to control) by selected compounds is presented in Table III. The most efficacious irreversible inhibitor in the series was carboxamide **5c**, which at a concentration of 20 nM irreversibly blocked >90% of the receptors during a 45-min incubation. Other very potent irreversible inhibitors were compound **22** and, to a lesser extent, compounds **5r** and **5d**.

SOLUBILITY OF ANALOGUES

The aqueous solubility of selected xanthines was measured by a spectrophotometric method (see the Experimental Procedures). The method was not applicable to the fluorescent analogues which contain nonxanthine chromophores that absorb in the range of 310 nm. The solubilities (micromolar) of selected derivatives are presented in Table IV. The derivatives with the greatest aqueous solubility (all greater than compound **2**) were found to be **5e**, **5g**, and **22**. Several of the results were unexpected, i.e. xanthines bearing groups that were predicted to enhance solubility instead resulted in greater insolubility. For example,

compound **5c**, which contains a carboxamido group, was only one-third as soluble as unsubstituted derivative **2**.

DISCUSSION

Previously, we demonstrated the feasibility of chemically cross-linking a functionalized congener, such as XAC or ADAC, to a receptor by prior monovalent reaction of the ligand with a bifunctional reagent (10, 11). Given the proximity to a nucleophile on the receptor, the remaining chemically reactive group of the congener was available for irreversible cross-linking during receptor binding. This approach afforded optimized, irreversible ligands that proved useful in characterizing the A₁ receptor in membrane preparations (11) and in isolated guinea pig hearts (12).

It became apparent from this earlier work that it would be desirable to incorporate additional substituents on the irreversible ligand for the purposes of altering physicochemical and pharmacological properties. A number of possibilities can be envisioned involving prosthetic groups, reporter labels, additional reactive centers, and water-solubilizing moieties. The principal goals of this project were to develop symmetrical trifunctional linkers and a flexible synthetic methodology which could permit a diverse array of derivatives to be prepared.

In design considerations, it was important to maintain symmetry in the central linker in order to exercise a degree of regiochemical control. This control could be achieved by having three equivalent reactive centers (such as the triisothiocyanatobenzene **21**) and sequentially adding the necessary chemical components or by incorporating a desired modifying group at the outset then generating two equivalent reactive sites (as in compound **14**) at a later stage of functionalization. The isothiocyanate group had proven to be an effective reactive group both to couple to the xanthine amine pharmacophore and to bind covalently to the A₁ adenosine receptor, as was shown previously (11). Therefore, at least two of the three sites in the linker were chosen to be isothiocyanates. Some variability of the linking moiety in the third site R' was needed to provide a logical means for attaching either a nucleophilic (in non-isothiocyanate intermediates) or electrophilic reagent for the desired chemical modification. For nucleophilic addition, the isothiocyanate group (leading to a thiourea) and succinimido active ester or acyl chloride (leading to a carboxamide) were chosen. To utilize the pool of commercially available amine reactive probes, a linker terminating in a free amine was developed (compound **8**). This range of reaction tuning permitted the permutability desired for different analogues.

Nonradioactive tracers, principally fluorescent-labeled ligands (13, 24), are under development as an alternative to the use of radioisotopes in receptor-binding assays and in histochemical studies. We now present an approach to the design of fluorescent ligands using trifunctional reagents that contain a reactive group for covalent attachment of the ligand to the receptor protein. Previously, glucocorticoid receptors, which unlike adenosine receptors are located intracellularly, were affinity labeled with a fluorescent group (25).

The identification of a class of potentially bioavailable, irreversible inhibitors of adenosine receptors may enable the study of receptors in systems where this was previously not possible. For example, in the trachea (26) 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 **22**. Inhibitors with improved water solubility, such as **5e**, may be useful in tissue or in vivo studies.

The central effects of adenosine (evidenced in locomotor depression, analgesia, protection against convulsions) had not been ascribed clearly to either A₁ or A₂ receptor subtypes. Recently however, the locomotor depression has been characterized pharmacologically as having both A₁ and A₂ components (27). Addition of compound **22** as a specific inhibitor in isolated brain preparations or in vivo may prove useful in the study of regulatory effects of endogenous adenosine and delineation of the role of A₁ and A₂ receptor subtypes. It may be necessary to administer the drug intracerebroventricularly for studies of the central nervous system, due to low rates of passage of complex purine derivatives across the blood–brain barrier and potential peripheral metabolism.

Irreversible inhibitors bearing fluorescent groups such as **5q** may be useful in histochemical studies. Radioisotopic labeled irreversible inhibitors, such as the radioactive equivalents of **5r**, may be useful in autoradiographic or imaging studies or in receptor assays. Due to possible nonspecific labeling in heterogeneous systems by the isothiocyanate group (11), these derivatives may be more useful with purified receptor preparations (9) than with tissue. Biotin conjugate **5n** would potentially be useful for all of the above applications and also for isolation of receptors on an avidin column (14). Several of the adenosine antagonists, such as **5k** and **5l**, bear chemically or photochemically reactive groups in addition to the *m*-isothiocyanate. These might be useful in studying peptide mapping or proximity of nucleophilic groups on the receptor protein, especially in combination with other protein-degradative techniques. Conceivably, this may be a means of cross-linking adjacent transmembrane regions of the receptor molecule. In the event that an additional chemically reactive group, such as the 5-isothiocyanate group of **22** or the bromoacetyl group of **5i**, would remain intact rather than react with the receptor protein, it conceivably could be utilized in a further reaction with a nucleophilic species added in high concentration externally. Such a “chemically functionalized” receptor would present versatile and unique possibilities for labeling or receptor isolation.

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Figure 1.
Synthesis of a class of irreversibly binding, high-affinity A₁ adenosine receptor antagonists (5) derived from trifunctional cross-linking reagents (4) present in molar excess.

Figure 2.

Routes of synthesis of trifunctional A₁ adenosine receptor inhibitors, in which the 5-position substituent (group R' in Table I) is linked through a carboxamide. Reagents: (a) MeOH, EDAC, 4-(dimethylamino)pyridine; (b) H₂N(CH₂)₂NH₂ (c) acylation with a succinimido ester or an isothiocyanate; (d) *N*-hydroxysuccinimide, EDAC; (e) RNH₂; (f) TFA; (g) H₂/Pd; (h) CSCI₂, base; (i) compound 1, excess Ar-NCS.

Figure 3. Routes of synthesis of trifunctional A₁ adenosine receptor inhibitors, in which the 5-position substituent (group R' in Table I) is linked through a thiourea. Reagents: (a–i) as in Figure 2, (j) Zn/AcOH.

Figure 4. Synthesis of an intermediate trifunctional cross-linking reagent, in which the 5-position substituent (group R' in Table I) consists of a hydroxymethyl, ester, or carboxylic anhydride group. Reagents: (a–j) as in Figures 2 and 3 (k) LiAlH_4 .

Figure 5. Concentration-dependent saturation of bovine cortical A₁ adenosine receptors with the radioligand [¹²⁵I]APNEA before and after treatment of the membranes with a xanthine isothiocyanate derivative (**22**): (top) incubation with 20 nM compound **22**, (bottom) incubation with 50 nM compound **22**. In each graph total binding before (●) and after (□) incubation is shown in comparison to nonspecific binding before (○) and after (×) incubation.

Table I
Synthesis of Trifunctional Cross-Linking Reagents and Other Intermediates^a and Xanthine Derivatives^b
Cross-Linkers and Intermediates (for structures refer to Figures 2–4)

compd	R''	% yield	mp, °C	formula	anal.
6		51	183–185	C ₁₇ H ₂₄ N ₂ O ₆	C, H, N
7		60	219–220 dec	C ₁₈ H ₂₆ N ₂ O ₆	C, H, N
8		87	216–217	C ₁₉ H ₃₀ N ₄ O ₅	C, H, N
9		67	217–218	C ₂₁ H ₂₇ N ₃ O ₈	H, N; C ^d
10a	(CH ₂) ₂ NHCOCH ₂ Br	99	160 ^a	C ₂₁ H ₃₁ N ₄ O ₆ Br	C, H; N ^f
10b	(CH ₂) ₂ NHCO(CH ₂) ₂ C ₆ H ₄ -4-OH	63	154–156	C ₂₈ H ₃₈ N ₄ O ₇	C, H, N
10c	(CH ₂) ₂ NH-biotin	65	154–158	C ₂₉ H ₄₄ N ₆ O ₇ S·DMF	C, H, N
10d	(CH ₂) ₂ NHCO-C ₆ H ₄ -4-CH ₂ Br	84	>250	C ₂₇ H ₃₅ N ₄ O ₆ Br·Et ₂ O	C, H, N
10e	(CH ₂) ₂ NHCO-C ₆ H ₃ -2-NO ₂ -5-N ₃	83	188–190 dec	C ₂₆ H ₃₂ N ₈ O ₈ ·0.2DMF·0.4H ₂ O	C, H, N
10f	(CH ₂) ₂ NHCO-C ₆ H ₃ -2-OH-4-N ₃	85	196–198 dec	C ₂₆ H ₃₃ N ₇ O ₇	C, H, N
10g	(CH ₂) ₂ NH-NBD	60	213–215 dec	C ₂₅ H ₃₁ N ₇ O ₈ ·0.5H ₂ O	C, H, N ^e
10h	(CH ₂) ₂ NH-FITC	51	>300	C ₄₀ H ₄₁ N ₅ O ₁₀ S·2DMF·2H ₂ O	C, H, N
10i	(CH ₂) ₂ NH-TRITC	71	>230	C ₄₄ H ₅₁ N ₇ O ₈ ·0.5H ₂ O	C, H, N
10j	(CH ₂) ₂ NH-Tempo	99	170–174 dec	C ₂₉ H ₄₇ N ₆ O ₆ S	C, H, N
12a	(CH ₂) ₂ NHCOCH ₃	62	204–205	C ₁₁ H ₁₂ N ₄ O ₆	C, H, N
12b	(CH ₂) ₂ N(CH ₃) ₂	44	oil	C ₁₁ H ₁₄ N ₄ O ₅ ·0.33H ₂ O	C, H, N
12c	(CH ₂) ₂ N(CH ₃) ₃ ⁺ I ⁻	96	233–235	C ₁₂ H ₁₇ N ₄ O ₅ I	C, H, N
13a	(CH ₂) ₂ NHCOCH ₂ Br	83	<i>b</i>	C ₁₁ H ₁₅ N ₄ O ₂ Br·TFA·H ₂ O	C, H, N
13b	(CH ₂) ₂ NH-biotin	99	<i>b</i>	C ₁₉ H ₂₈ N ₆ O ₃ S·2.5TFA·H ₂ O	C, H, N
13c	(CH ₂) ₂ NHCO-C ₆ H ₃ -2-OH-4-N ₃	80	122–124	C ₁₆ H ₁₇ N ₇ O ₃ ·1.5TFA	C, H, N
13d	(CH ₂) ₂ NH-Tempo	70	155–160 dec	C ₁₉ H ₃₁ N ₆ O ₂ S·3TFA	C, H, N
14a	H	87	152–153	C ₉ H ₅ N ₃ OS ₂	C, H, N
14b	(CH ₂) ₂ NHCOCH ₃	67 ^c	190–192	C ₁₃ H ₁₂ N ₄ O ₂ S ₂ ·EtOAc·1.25H ₂ O	C, H, N
14c	(CH ₂) ₂ NHCOCH ₂ Br	74	185–188	C ₁₃ H ₁₁ N ₄ O ₂ BrS ₂	H, N; C ^{g,h}

Cross-Linkers and Intermediates (for structures refer to Figures 2-4)

compd	R''	% yield	mp, °C	formula	anal.
14d	(CH ₂) ₂ N(CH ₃) ₂ HCl	49 ^c	170–172 dec	C ₁₃ H ₁₅ N ₄ OClS ₂	C, H, N
14e	(CH ₂) ₂ NHCO(CH ₂) ₂ -C ₆ H ₄ -4-OH	56	>270	C ₂₀ H ₁₈ N ₄ O ₃ ·H ₂ O	C, H, N ^d
14f	(CH ₂) ₂ NH-biotin	67	>270	C ₂₁ H ₂₄ N ₆ O ₃ S ₂	<i>h</i>
14g	(CH ₂) ₂ NHCO-C ₆ H ₃ -2-NO ₂ -5-N ₃	40	146–150 dec	C ₁₈ H ₁₂ N ₈ O ₄ S ₂	<i>h</i>
14h	(CH ₂) ₂ NHCO-C ₆ H ₃ -2-OH-4-N ₃	73	150–152	C ₁₈ H ₁₃ N ₇ O ₃ S ₂	<i>h</i>
14i	(CH ₂) ₂ NH-NBD ₃	50	215–220	C ₁₇ H ₁₁ N ₇ O ₄ S ₂	<i>h</i>
14j	(CH ₂) ₂ NH-FITC	33	>240	C ₃₂ H ₂₁ N ₅ O ₆ S ₃	<i>h</i>
16		89	176–178	C ₇ H ₃ N ₃ O ₄ S	C, H, N
17a	(CH ₂) ₂ NHCOCH ₃	92	212–214	C ₁₁ H ₁₃ N ₃ O ₃ S	C, H, N
17b	(CH ₂) ₂ N(CH ₃) ₂	14	168–170	C ₁₁ H ₁₅ N ₅ O ₄ S	C, H, N
19a	(CH ₂) ₂ NHCOCH ₃	35	148–149	C ₁₃ H ₁₃ N ₅ OS ₃	C, H, N
19b	(CH ₂) ₂ N(CH ₃) ₂	41	>250	C ₁₅ H ₁₅ N ₅ S ₃	<i>h</i>
19c	CH ₂ CO ₂ C(CH ₃) ₃	28	137–139	C ₁₃ H ₁₆ N ₄ O ₂ S ₃	C, H, N
21		84	64–66	C ₉ H ₃ N ₃ S ₃	C, H, N
22		81	171–175 dec	C ₃₀ H ₃₁ N ₉ O ₄ S ₃ ·0.5H ₂ O	C, H, N
24		26	160–161	C ₁₈ H ₆ N ₂ O ₃ S ₄	C, H, N
26		96	74–75	C ₁₁ H ₈ N ₂ O ₂ S ₂	C, H, N
27		65	108–110	C ₇ H ₁₀ N ₂ O	C, H, N
28		63	79–80	C ₉ H ₆ OS ₂	C, H, N

Xanthine Derivatives

compd	R'	% yield	formula	anal.
5a	CO ₂ CH ₂ CH ₃	93	C ₃₂ H ₃₆ N ₈ O ₆ S ₂ ·DMF·0.5H ₂ O	C, H, N
5b	CH ₂ OH	94	C ₃₀ H ₃₃ N ₈ O ₅ S ₂ ·H ₂ O	C, H, N

Cross-Linkers and Intermediates (for structures refer to Figures 2–4)

compd	R ^{''}	% yield	mp, °C	formula	anal.
5c	CONH ₂		77	C ₃₀ H ₃₃ N ₉ O ₅ S ₂ ·DMF·H ₂ O	C, H, N
5d	CONH(CH ₂) ₂ N(CH ₃) ₂		79	C ₃₄ H ₄₂ N ₁₀ O ₅ S ₂ ·HCl·2.5H ₂ O	C, H, N
5e	CONH(CH ₂) ₂ NHCOCH ₃		73	C ₃₄ H ₄₀ N ₁₀ S ₂ ·DMF·3H ₂ O·HOAc	C, N; H ^k
5f	NHCSNH(CH ₂) ₂ N(CH ₃) ₂		27	C ₃₄ H ₄₃ N ₁₁ O ₄ S ₃	H, N; C ^l
5g	NHCSNH(CH ₂) ₂ NHCOCH ₃		33		
5h	NHCSNHCH ₂ CO ₂ C(CH ₃) ₃		64	C ₃₂ H ₃₆ N ₈ O ₆ S ₂ ·5H ₂ O	C, N; H ^m
5i	CONH(CH ₂) ₂ NHCOCH ₂ Br		74	C ₃₄ H ₃₉ N ₁₀ O ₆ BrS ₂	C, H, N
5k	NHCSNH(CH ₂) ₂ NHCO-C ₆ H ₃ -2-NO ₂ -5-N ₃		25	C ₃₂ H ₃₆ N ₈ O ₆ S ₂ ·DMF·2H ₂ O·HOAc	C, H, N
5l	NHCSNH(CH ₂) ₂ NHCO-C ₆ H ₃ -2-OH-4-N ₃		30	C ₃₂ H ₃₆ N ₈ O ₆ S ₂ ·DMF·2H ₂ O·4HOAc	C, H, N
40	<i>n</i>		72	C ₂₈ H ₃₁ N ₉ O ₄ S ₂ ·0.5H ₂ O	C, H, N

Abbreviations:

biolin =

-NBD =

-TRITC =

-Tempo =

^aTemperature raised rapidly, decomposes.

^bAmorphous solid.

^cVia compound **12**, % yield from compound **13**.

^dCalcd 56.12, found 56.62.

^eCalcd 17.31, found 16.36.

^fCalcd 10.87, found 11.61.

^gCalcd 39.11, found 40.58.

^hAccurate mass determined using fast atom bombardment mass spectroscopy (calculated mass for m + 1 in parentheses): **14c** + 0.2 ppm (399.9487) for ⁸¹Br isotopic form; **14e** – 0.1 ppm (427.0899); **14f** + 0.8 ppm (505.1150); **14g** + 0.1 ppm (469.0501); **14h** + 0.0 ppm (440.0600); **14i** + 1.7 ppm (442.0392); **14j** – 0.6 ppm (668.0732); **19b** molecular ion + 0.5 ppm (338.0568) and fragment corresponding to loss of (CH₃)₂NH + 0.7 ppm (292.9989).

ⁱFor complete structures and methods of preparation refer to Figures 2–4. Compounds **14** were synthesized via **8** and **10**, unless noted. Compounds **19** were synthesized from **21**. Compound **12c** was synthesized from **12b** by methylation.

^jCompounds **5f**, **5g**, and **5h** were synthesized via structure **19**, and compounds **5k**, **5l**, **5o**, **5p**, and **5r** were synthesized via compound **22**.

^kCalcd H 6.14%, found 4.83.

^lCalcd. 53.32, found 48.15.

^mCalcd. 6.05, found 5.42.

ⁿFor structure refer to Table 2.

Receptor Affinities of Xanthine Derivatives, Expressed as the K_i Values in nM, for Inhibition of Binding of [3 H]PIA at A_1 Receptors in Rat Cerebral Cortical Membranes, or for Inhibition of Binding of [3 H]CGS21680 at A_2 Receptors in Rat Striatal Membranes

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compd	R''' ^a	apparent K_i at central rat adenosine receptors, nM		
		A ₁	A ₂	A ₂ /A ₁ ratio
5j		45.0 ± 3.5	493 ± 200	11.0
5k	NHCSNH(CH ₂) ₂ NHCO-C ₆ H ₃ -2-NO ₂ -5-N ₃	27.3 ± 7.2	851 ± 40	31.2
5l	NHCSNH(CH ₂) ₂ NHCO-C ₆ H ₃ -2-OH-4-N ₃	30.1 ± 7.6	353 ± 93	11.7
5m	CONH(CH ₂) ₂ NHCO(CH ₂) ₂ -C ₆ H ₄ -4-OH	7.08 ± 1.91	1400 ± 330	198
5n	CONH(CH ₂) ₂ NH-biotin	20.1 ± 3.3	10700 ± 1300	532
5o	NHCSNH(CH ₂) ₂ NH-Tempo	52.1 ± 16.4	593 ± 230	11
5p	NHCSNH(CH ₂) ₂ NH-FITC	234 ± 33	2130 ± 480	9.13
5q	CONH(CH ₂) ₂ NH-NBD	22.3 ± 7.2	1310 ± 220	58.7
5r	NHCSNH(CH ₂) ₄ NHCO-C ₆ H ₄ -4-CH ₂ F	43.6 ± 12.3	593 ± 135	13.6

^aTEMPO = 2,2,6,6-tetramethyl-1-piperidinyloxy, NBD = 4-nitrobenz-2-oxa-1,3-diazole, FITC = fluorescein isothiocyanate, for structures refer to Table I.

^bNot determined.

^cVersus [³H]NECA.

Table III

Inhibition of [125 I]APNEA Binding to A₁ Adenosine Receptors in Bovine Brain Membranes by Xanthine Isothiocyanate Derivatives^a

compd	IC ₅₀ ^b	concn, nM	% decrease ^c
2	nd	5	49 ^d
		50	67 ^d
		500	94 ^d
5c	nd	5	68.0 ± 7.5 (3)
		20	90.3 ± 7.0 (3)
5d	nd	20	30.0 ± 8.7 (3)
		50	46.3 ± 21.8 (6)
5h	30.2	100	13 (1)
		250	23 (1)
5i	96.4	250	23 (2)
5k	116	50	0 (2)
		250	13 (2)
5k	22.9	250	34 (3)
5m	nd	250	18 (2)
5n	nd	500	50 (1)
5o	53.5	250	29 (1)
5p	85.7	250	15 (2)
5q	32.9	50	21 (2)
		100	41 (2)
5r	36.5	50	43 (2)
22	nd	20	30.3 ± 12.2 (3)
		50	66.0 ± 19.7 (3)

^aIncubation (45 min) with compound followed by exhaustive washout and radioligand saturation experiment.

^bIn competition binding assay. nd = not determined.

^cPercent decrease in density of A₁ adenosine receptors ($\Delta B_{\max}$ relative to control) ± standard deviation for three or more experiments. The number of experiments is given in parentheses.

^dData from ref 10.

Table IV

Maximum Solubilities of Selected Xanthine Isothiocyanate Derivatives in Aqueous Buffer (pH 7.2)

compd	solubility, ^a μ M	compd	solubility, ^a μ M
2	90.1 $\pm$ 0.6	5f	5.07 $\pm$ 0.73
5a	9.33 $\pm$ 0.43	5g	>100
5b	10.8 $\pm$ 0.0	5p	29.2 $\pm$ 0.8
5c	31.4 $\pm$ 0.4	22	112 $\pm$ 2
5d	28.7 $\pm$ 0.0	40	17.5 $\pm$ 0.1
5e	>1000		

^aConcentration measured by UV absorbance at 310 nm after transfer of an aqueous aliquot to methanolic solution.