Supporting Information for *ChemBioChem* F404

Receptor-Dependence of the Transcription Read-Out in a Small-Molecule Three-Hybrid System**

Wassim M. Abida, Brian T. Carter,
Eric A. Althoff, Hening Lin, and Virginia W. Cornish

Chemical synthesis

General methods

Unless otherwise noted, reagents were obtained from Aldrich and used without further purification. Dexamethasone was purchased from Sigma; benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate (PyBOP), from Advanced ChemTech; and L-homocystine, from Fluka. Anhydrous *N*,*N*-dimethylformamide and anhydrous methylene chloride were from Sure Seal™ bottles purchased from Aldrich. All moisture- or oxygen-sensitive reactions were performed under a positive pressure of nitrogen in flame- or oven-dried glassware. Organic extracts were dried over magnesium sulfate or sodium sulfate. Organic solvents were removed *in vacuo* with a rotary evaporator equipped with a vacuum pump (ca. 1 torr). Products obtained as solids or oils were dried under vacuum (ca. 1 torr).

Analytical thin layer chromatography (TLC) was performed on silica gel (Whatman LHPKF Silica Gel 60Å) and visualized by UV light (254 nm) and staining with cerium molybdate or ninhydrin. All column chromatography was flash chromatography carried out on silica gel (EM Science Silica Gel 60), and all eluants used are reported in volume:volume ratios.

Melting points (Pyrex capillary) are uncorrected. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker 400

(400 MHz) or a Bruker 300 (300 MHz) Fourier Transform (FT) NMR spectrometer at the Columbia University Department of Chemistry NMR facility. Spectra were determined in chloroform-*d* with TMS (0δ) or the proton or carbon (7.25δ; 77.0δ) as the reference; in methanol-d_4 with the methyl protons or carbon (3.30δ; 49.0δ) as the reference; or in dimethyl sulfoxide-d_6 (DMSO-d_6) with the protons or carbons (2.49δ; 39.5δ) as the reference. ^{1}H NMR resonances are reported in units of parts per million (ppm) downfield from trimethylsilane and are tabulated in the following order: multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad), coupling constant(s) in Hertz (Hz), number of protons. ^{13}C NMR spectra were determined at 75 MHz on the Bruker 300 MHz instrument; all ^{13}C NMR spectra are proton decoupled. Infrared (IR) spectra were recorded on a Perkin Elmer 1600 series FT IR spectrometer using sodium chloride (NaCl) plates or potassium bromide (KBr) pellets. Mass spectra (MS) were recorded at the Columbia University Department of Chemistry Mass Spectral laboratory. Low resolution electron spray ionization (ESI) MS were recorded on a JMS-LC mate mass spectrometer. Fast Atom Bombardment (FAB) high resolution mass spectra (HRMS) were recorded on a JMS-HX110A mass spectrometer.

*Synthesis of **4** and **9***

We have previously described the syntheses of the thiol derivative of dexamethasone (4) and the thiol precursor to methotrexate (9).[1]

Synthesis of 2,4-diamino-6-bromomethyl pteridine

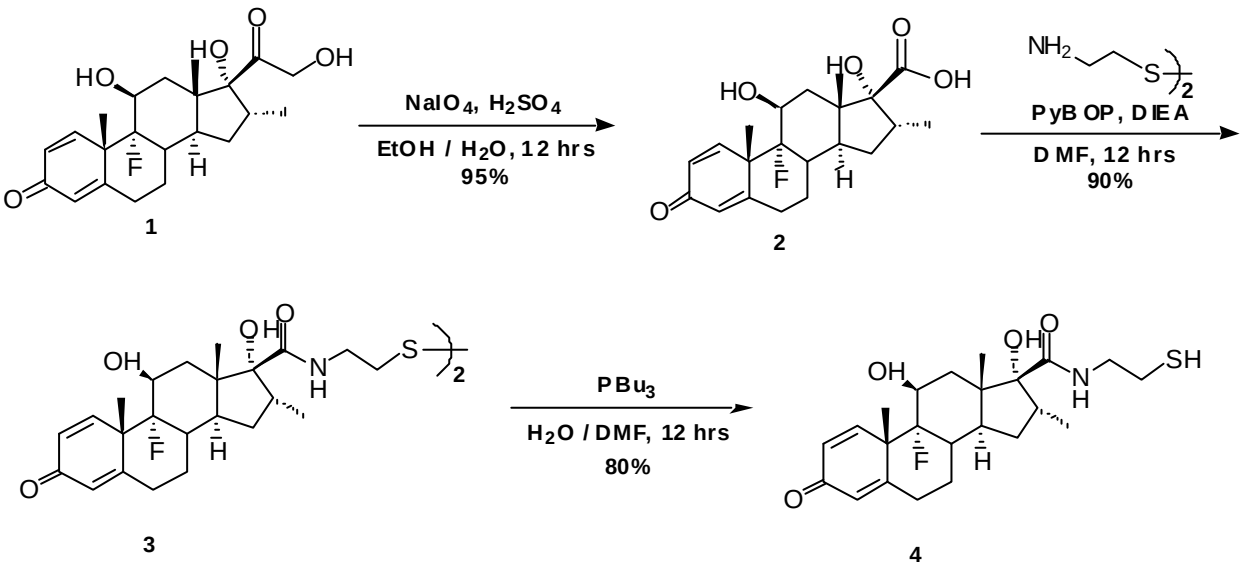
The synthesis of 2,4-diamino-6-bromomethyl pteridine has been described in the literature.[29, 30] Here we report our detailed protocol. 2,4,5,6-Tetraaminopyrimidine sulfate (4.77 g, 20.0 mmol) was suspended in water (80 mL), stirred in boiling water bath for 10 minutes (min) to give an almost clear yellow solution. Barium chloride (4.89 g, 20.0 mmol) in water (20 mL) was added to the solution. The mixture was stirred in the boiling water bath for an additional 10 min. After cooling down, the barium sulfate precipitate was removed by filtration and the solid was washed with cold water (50 mL). Water (150 mL) was added to the combined washings and filtrate to make the volume of the solution 300 mL. In a 2 liter Erlenmeyer flask, 1,3-dihydroxyacetone (5.40 g, 60.0 mmol) and cysteine hydrochloride monohydrate (3.15 g, 20.0 mmol) were added to 4 M sodium acetate aqueous solution (300 mL). The pH of this solution was then adjusted to 9 with 1 N NaOH. To this pH = 9 solution, the 300 mL 2,4,5,6-tetraaminopyrimidine solution was added. The resulting reaction mixture was bubbled with air at a

rate of approximately 1 mL/sec and stirred at room temperature for 24 hours (hr). After this, the flask was placed on ice for 2 hrs. The precipitate was collected by filtration and washed with cold water (20 mL), then ethanol (30 mL), and finally ether (50 mL). The dried crude product was pulverized and put in a 500 mL flask. Hot ethanol (200 mL) was added. The suspension was boiled and stirred in a 100^{o}C bath. A solution of 48% HBr in ethanol (1:18 in volume) was added slowly until most of the solid dissolved. The solution was filtered while hot and the filtrate was kept in a refrigerator overnight while a first crop of 2, 4-diamino-6-hydroxylmethyl pteridine hydrobromide salt separated. A second crop was obtained after concentration of the filtrate to one third of the original volume. The solid was washed with ether and dried (45% yield). ^{1}H NMR (300 MHz, methanol-d_4) δ 8.93 (s, 1), 4.91 (s, 2); MS, m/z 193.1 (MH^{+}). 2, 4-diamino-6-hydroxylmethyl pteridine hydrobromide (3 g, 11.0 mmol) was added to Ph_3PBr_2 (14.3 g, 36.3 mmol) in anhydrous N,N'-dimethylacetamide (360 mL). The reaction mixture was stirred at room temperature for 3.5 hrs. The solution that formed was treated with ethanol (1 mL) and stirred for 15 min longer before benzene (1200 mL) was added. A dark oil precipitated, and the mixture was stirred for 30 min and left to stand overnight. The clear supernatant was siphoned and decanted form the now semisolid precipitate, to which hot acetic acid was added until

most of the solid dissolved. The solution was filtered while hot. The solid that separated from the cooled filtrate was collected after 4 hrs at room temperature (rt). The solid was washed with ether (20 mL), dried in air and then recrytallized from 2-propanol, washed with ether (20 mL) and dried in air. ^{1}H-NMR (300 MHz, methanol-d_4) δ 8.98 (s, 1), 4.83 (s, 2), 3.93 (m, J = 6.1, ~1), 1.17 (d, J = 6.1, ~6); MS m/z 255.0, 257.0 (M^+).

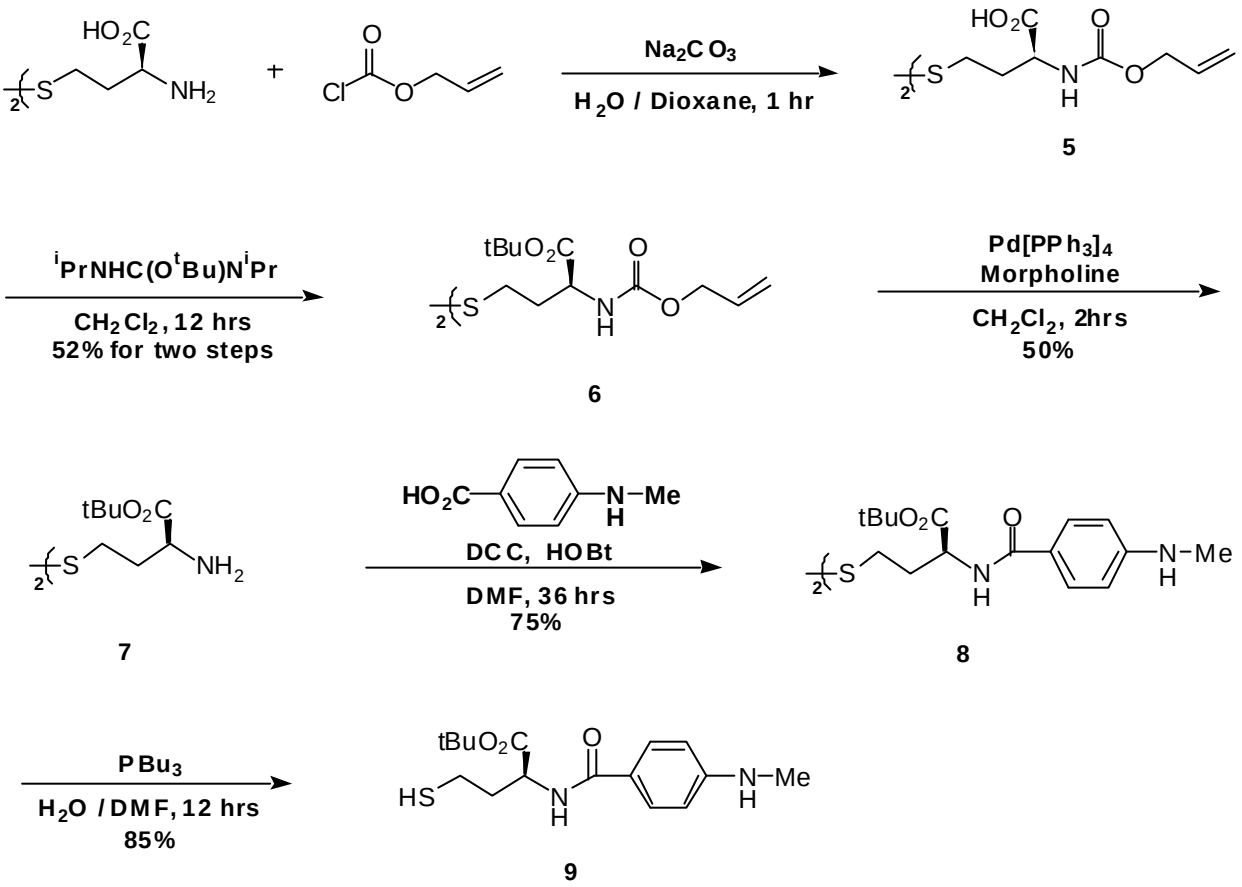
Scheme 1

A



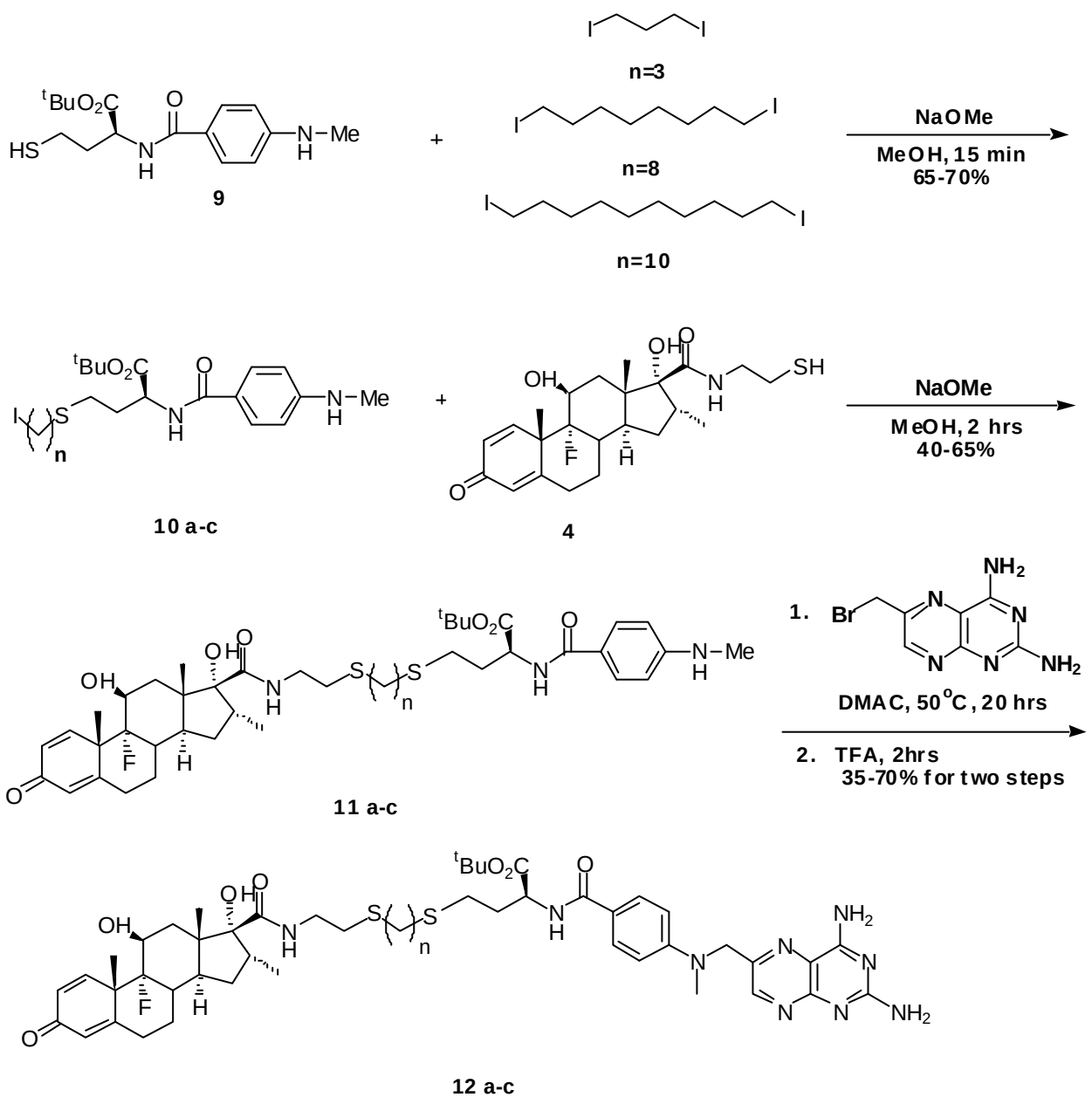
Scheme 1 cont'd

B



Scheme 1 cont'd

c



Synthesis of 10a-c

Compound 9 (441 mg, 1.36 mmol) and 1,10-diiododecane (2.67 g, 6.78 mmol) were dissolved in methanol (1.4 mL). The reaction was purged with nitrogen, and then a 0.50 M solution of sodium methoxide in methanol (3.25 mL, 1.63 mmol) was added to the reaction mixture. The reaction was stirred at rt for 15 min and then concentrated *in vacuo*. The product was purified by silica chromatography using 3:1 hexanes:ethyl acetate to give 551 mg (69% yield) of a white solid: R_f = 0.75 in 10:1 methylene chloride:methanol; m.p., 73-74 °C; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.70 (d, J = 9.0 Hz, 2), 6.61 (d, J = 9.0 Hz, 2), 4.65 (dd, J = 5.0, 9.0 Hz, 1), 3.24 (t, J = 7.0 Hz, 2), 2.83 (s, 3), 2.70-2.58 (m, 2), 2.55 (t, J = 7.0 Hz, 2), 2.17-2.07 (m, 2), 1.84-1.77 (m, 3), 1.69-1.59 (m, 2), 1.50 (s, 9), 1.41-1.31 (m, 13); ^{13}C NMR (75 MHz, methanol-d_4) δ 172.2, 169.6, 153.6, 129.2, 120.7, 111.0, 81.8, 53.1, 48.6, 48.3, 48.0, 47.7, 47.4, 47.2, 33.7, 31.8, 31.7, 30.5, 29.7, 29.5, 29.3, 29.0, 28.8, 28.6, 28.3, 27.3; IR 3401, 3378, 2980, 2924, 2850, 2530, 2368, 1732, 1637, 1609, 1576, 1532, 1516, 1449, 1337, 1336, 1308, 1287, 1238, 1190, 1165, 1107, 984, 837, 766, 697, 597 cm^{-1}; MS, m/z 591.3 (MH^+); HRMS, calculated 591.2117, found 591.2111 (MH^+).

Compound 10b was prepared essentially as described for compound 10a to give 492 mg (71% yield) of the desired product:

R_f = 0.65 in 1:1 hexanes:ethyl acetate; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.70 (d, J = 9.0 Hz, 2), 6.61 (d, J = 9.0 Hz, 2), 4.63 (dd, J = 9.0, 5.0 Hz, 1), 3.28 (t, J = 7.0 Hz, 2), 2.83 (s, 3), 2.72-2.55 (m, 2), 2.52 (t, J = 7.0 Hz, 2), 2.20-2.00 (m, 2), 1.78 (p, J = 7.0 Hz, 2), 1.58 (p, J = 7.0 Hz, 2), 1.50 (s, 9), 1.45-1.33 (m, 4), 1.33-1.25 (m, 4); MS, m/z 563.3 (MH^+)

Compound 10c was prepared essentially as described for compound 10a to give 191 mg (75% yield) of the desired product: R_f = 0.55 in 1:1 hexanes:ethyl acetate; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.70 (d, J = 9.0 Hz, 2), 6.61 (d, J = 9.0 Hz, 2), 4.63 (dd, J = 9.0, 5.0 Hz, 1), 3.40 (t, J = 7.0 Hz, 2), 2.83 (s, 3), 2.72-2.55 (m, 2), 2.66 (t, J = 7.0 Hz, 2), 2.25-2.00 (m, 2), 2.05 (t, J = 7.0 Hz, 2), 1.50 (s, 9); MS, m/z 493.3 (MH^+)

Synthesis of 11a-c

Compound 10a (80 mg, 0.14 mmol) and the thiol derivative of Dex (4) (71 mg, 0.16 mmol) were dissolved in methanol (0.5 mL), and then the reaction mixture was purged with nitrogen. A 0.50 M solution of sodium methoxide in methanol (0.33 mL, 0.16 mmol) was added to the reaction mixture, and the resulting solution was stirred at rt for 2 hrs. The reaction was concentrated *in vacuo*. The product was purified by silica chromatography using 50:1 methylene chloride:methanol followed by 40:1 methylene chloride:methanol to give 80 mg (66% yield) of a white solid: R_f

= 0.60 in 10:1 methylene chloride:methanol; m.p., 77-78 °C; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.73 (d, J = 9.0 Hz, 2), 7.44 (d, J = 10.0 Hz, 1), 6.61 (d, J = 9.0 Hz, 2), 6.30 (d, J = 10.0, 1), 6.09 (s, 1), 4.69-4.63 (m, 1), 4.25 (d, J = 11.0 Hz, 1), 3.54-3.46 (m, 1), 3.33 (t, J = 2.0 Hz, 3), 3.18-3.09 (m, 1), 2.84 (s, 3), 2.76-2.50 (m, 8), 2.45-2.36 (m, 2), 2.24-2.03 (m, 4), 1.94-1.75 (m, 8), 1.61-1.31 (m, 28), 1.25-1.17 (m, 1), 1.12 (s, 3), 0.92 (d, J = 7.0 Hz, 3); ^{13}C NMR (75 MHz, methanol-d_4) δ 187.7, 174.6, 172.1, 169.7, 169.5, 154.8, 153.6, 129.3, 128.9, 124.2, 120.7, 110.9, 102.9, 100.4, 87.2, 81.6, 72.3, 71.9, 53.1, 49.5, 48.9, 48.6, 48.3, 48.1, 47.8, 47.5, 47.2, 43.9, 39.0, 35.9, 35.3, 35.0, 34.7, 32.5, 32.0, 31.9, 31.8, 29.8, 29.7, 29.4, 28.9, 27.4, 26.5, 24.3, 24.1, 23.8, 16.9, 14.3, 13.0; IR 3442.6 (broad), 3062, 2928, 2855, 2360, 2341, 1810, 1731, 1663, 1607, 1518, 1491, 1446, 1366, 1319, 1279, 1239, 1192, 1155, 1079, 1033, 1012, 892, 833, 791, 760, 700, 668, 638, 588 cm^{-1}; MS, m/z 900.5 (MH^+); HRMS, calculated 900.5030, found 900.5022 (MH^+).

Compound 11b was prepared essentially as described for compound 11a to give 564 mg (74% yield) of the desired product: R_f = 0.60 in 10:1 methylene chloride:methanol; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.70 (d, J = 9.0 Hz, 2), 7.42 (d, J = 10.0 Hz, 1), 6.61 (d, J = 9.0 Hz, 2), 6.29 (dd, J = 10.0, 2.0 Hz,1), 6.10 (s, 1), 4.63 (dd, J = 9.0, 5.0 Hz, 1), 4.23 (d, J = 11.0 Hz,

1), 3.60-3.39 (m, 3), 3.15 (m, 1), 2.83 (s, 3), 2.82-2.63 (m, 4), 2.63-2.53 (m, 4), 2.53-2.30 (m, 2), 2.22-2.00 (m, 4), 1.94-1.84 (m, 1), 1.83-1.63 (m, 2), 1.61 (s, 3), 1.60-1.41 (m, 8), 1.50 (s, 9), 1.50-1.40 (m, 5),1.23 (m, 1), 1.10 (s, 3), 0.90 (d, J = 7.0 Hz, 3); MS, m/z 872.6 (MH^+), 873.6 (MH_2^+)

Compound 11c was prepared essentially as described for compound 12a to give 44 mg (31% yield) of the desired product: R_f = 0.50 in in 10:1 methylene chloride:methanol; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.70 (d, J = 9.0 Hz, 2), 7.42 (d, J = 10.0 Hz, 1), 6.61 (d, J = 9.0 Hz, 2), 6.29 (dd, J = 10.0, 2.0 Hz,1), 6.10 (s, 1), 4.63 (dd, J = 9.0, 5.0 Hz, 1), 4.23 (d, J = 11.0 Hz, 1), 3.49 (m, 2), 3.15 (m, 1), 2.83 (s, 3), 2.72-2.59 (m, 8), 2.53-2.30 (m, 2), 2.22-2.00 (m, 5), 1.94-1.78 (m, 2), 1.74 (q, J = 11.0 Hz, 2), 1.61 (s, 3), 1.60-1.51 (m, 2), 1.50 (s, 9), 1.23 (m, 1), 1.10 (s, 3), 0.90 (d, J = 7.0 Hz, 3); MS, m/z 802.2 (MH^+), 803.2 (MH_2^+)

Synthesis of 12a-c

Compound 11a (96 mg, 0.11 mmol) and the hydrobromide salt of 2,4-diamino-6-bromomethyl pteridine (46 mg, 0.12 mmol) were dissolved in *N,N*-dimethylacetamide (0.36 mL). The reaction flask was placed in an oil bath, and then the mixture was stirred at 50°C for 12 hrs. The reaction was concentrated *in vacuo*. The protected intermediate (R_f = 0.36 in 10:1 methylene

chloride:methanol) was separated by silica chromatography using a gradient of 30:1 to 20:1 methylene chloride:methanol. This crude intermediate then was dissolved in trifluoroacetic acid (6.5 mL) and stirred at rt for 1 hr. The trifluoroacetic acid was removed *in vacuo* by azeotroping with toluene (3x, 25 mL). The final product (compound 12a) was purified by silica chromatography using a gradient of 10:1 to 2:1 methylene chloride:methanol to give 70 mg (65% overall yield for two steps) of a yellow solid: R_f = 0.25 in 90:15:1 chloroform:methanol:triethyl amine; ^{1}H NMR (400 MHz, methanol-d_4) δ 8.56 (s, 1), 7.78 (d, J = 9.0 Hz, 2), 7.44 (d, J = 10.0 Hz, 1), 6.89 (d, J = 9.0 Hz, 2), 6.30 (d, J = 10.0, 1), 6.09 (s, 1), 4.57-4.52 (m, 2), 4.23 (d, J = 11.0 Hz, 1), 3.50-3.43 (m, 1), 3.39-3.23 (m, 4), 3.18-3.06 (m, 1), 2.79-2.33 (m, 10), 2.10, 2.02 (m, 1), 2.26-2.15 (m, 3), 2.00-1.70 (m, 9), 1.60-1.47 (m, 10), 1.38-1.24 (m, 16), 1.10 (s, 3), 0.91 (d, J = 7.0, 3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 186.2, 173.1, 168.2, 166.4, 163.7, 156.0, 153.9, 151.7, 150.1, 146.0, 129.8, 129.6, 125.0, 122.6, 122.1, 112.1, 103.6, 101.2, 87.3, 72.0, 71.4, 55.9, 49.0, 48.8, 48.1, 43.8, 41.5, 41.4, 41.3, 41.2, 41.1, 40.0, 39.8, 36.3, 35.2, 34.9, 34.7, 33.5, 32.8, 31.9, 31.8, 31.7, 31.2, 29.9, 29.8, 29.4, 29.1, 28.7, 28.3, 23.9, 17.9, 16.0; IR 3424.9 (broad), 2929, 2364, 2344, 1654, 1637, 1607, 1560, 1542, 1508, 1448, 1420, 1364, 1297, 1244, 1206, 1138, 1034, 1014, 984, 890, 835,

769 cm^{-1}; MS, m/z 1018.5 (MH^+); HRMS is not reported because of its inaccuracy at MW > 1000.

Compound 12b was prepared essentially as described for compound 12a to give 236 mg (67 % yield for two steps) of the desired product: R_f (intermediate) = 0.25 in 10:1 methylene chloride:methanol; R_f (final product) = 0.15 in 4:1 methylene chloride:methanol; ^1H NMR (400 MHz, methanol-d_4) δ 8.59 (s, 1), 7.77 (d, J = 9.0 Hz, 2), 7.42 (d, J = 10.0 Hz, 1), 6.89 (d, J = 9.0 Hz, 2), 6.29 (dd, J = 10.0, 1.8 Hz,1), 6.09 (s, 1), 4.75 (s, 2), 4.60 (m, 1), 4.20 (d, J = 8.0 Hz, 1), 3.65-3.4 (m, 3), 3.25 (s, 3), 3.10 (m, 1), 2.67-2.47 (m, 6), 2.47-2.25 (m, 2), 2.25-2.12 (m, 3), 2.08 (m, 1), 2.08-2.00 (m,1), 1.94-1.72 (m, 4), 1.61 (s, 3), 1.60-1.51 (m, 2), 1.40-1.20 (m, 12), 1.10 (s, 3), 0.90 (d, J = 7.0 Hz, 3); MS, m/z 991.3 (MH^+).

Compound 12c was prepared essentially as described for compound 12a to give 12 mg (67 % yield for two steps) of the desired product: R_f (intermediate) = 0.20 in 10:1 methylene chloride:methanol; R_f (final product) = 0.35 in 3:1 methylene chloride:methanol; ^1H NMR (400 MHz, methanol-d_4) δ 8.59 (s, 1), 7.77 (d, J = 9.0 Hz, 2), 7.42 (d, J = 10.0 Hz, 1), 6.89 (d, J = 9.0 Hz, 2), 6.29 (dd, J = 10.0, 2.0 Hz,1), 6.09 (s, 1), 4.71 (s, 2), 4.50 (dt, J = 9.0, 5.0 Hz, 1), 4.20 (d, J = 8.0 Hz, 1), 3.69 (s,1), 3.65-3.4 (m, 2), 3.26 (s, 3), 3.10 (m, 1), 2.72 (m, 1), 2.67-2.52 (m, 8), 2.53-2.30 (m, 2), 2.32-2.15 (m, 2), 2.08 (m,

1),1.94-1.78 (m, 4), 1.61 (s, 3), 1.60-1.51 (m, 2), 1.20 (m, 1), 1.10 (s, 3), 0.90 (d, J = 7.0 Hz, 3); MS, m/z 920.3 (MH^+), 921.3 (MH_2^+).

Molecular biology

General methods for molecular biology

Restriction enzymes, Vent DNA polymerase and T4 DNA ligase were purchased from New England Biolabs. The dNTPs used in the Polymerase Chain Reation (PCR) were purchased from Pharmacia Biotech. Oligonucleotides were purchased from The Great American Gene Company (www.geneco.com). The bacto-agar, bacto-peptone and bacto-yeast extract were purchased from DIFCO. Corning Costar 96-well plates with V-shaped wells used for growing yeast and Corning Costar 96-well UV-plates used in liquid assays were purchased from Fisher. The phrog used to transfer cells into 96-well plates or onto petri plates containing agar media was purchased from Dan-Kar Corp. (Wilmington, MA). Single stranded DNA (DNA sodium salt type III, salmon testes) used for yeast transformation was purchased from Sigma. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for the plate assays was purchased from Diagnostic Chemicals (Oxford, CT). o-Nitrophenyl-β-D-galactopyranoside (ONPG) for the liquid assays was purchased from Sigma.

Methotrexate was from the National Cancer Institutes (NCI). All other chemicals were purchased from Aldrich or Sigma.

Detection of fusion proteins was performed using a Phototope®-HRP Western Blot Detection Kit (Cell Signaling Technologies). Total protein concentration was assessed by the Bradford method with bovine serum albumin (BSA) as a standard using a kit purchased from Bio-Rad. *N,N,N',N'*-Tetramethylethylenediamine (TEMED) and Acrylamide:Bis-Acrylamide (37.5:1) for making 12% Acrylamide/Bis-Acrylamide gels were purchased from Fisher. For protein molecular weight detection, the low range, biotinylated SDS-PAGE standards kit from Bio-Rad was used. Proteins were transferred from polyacrylamide gels to Immobilon-P 0.45 μm polyvinylidene fluoride (PVDF) membranes (Millipore) for Western blot detection. LexA-containing fusion proteins were detected using an anti-LexA monoclonal antibody purchased from Clontech. Proteins containing a hemoglutanin (HA) epitope tag were detected using anti-HA 12CA5 antibody purchased from Boehringer Mannheim. PVDF membrane was blocked with Carnation nonfat dry milk (Nestle®). Hyperfilm MP autoradiography film was purchased from Amersham Pharmacia Biotech.

All aqueous solutions were made with distilled water prepared from a Hydro Ultrapure Water System. For Polymerase

Chain Reaction (PCR), a MJ Research PTC-200 Pellier Thermal Cycler was employed. The transformation of *E. coli* was carried out by electroporation using a Bio-Rad *E. coli* Pulser. The liquid assays were carried out using an HTS 7000 Plus BioAssay Reader from Perkin Elmer. A M35A-M X-OMAT Processor from Kodak was used to develop the film for the Western blot detections. Restriction digests were carried out as recommended by New England Biolabs. Sequencing of the receptor domains of all plasmids constructed was performed by Gene Wiz (New York, NY). All yeast techniques including the preparation of yeast media were carried out following standard protocols.[27] All other standard molecular biology techniques were carried out essentially as described.[31, 32]

The small molecules were dissolved in DMF to concentrations of 10 mM for Dex and Mtx and 5 mM for the Dex-Mtx molecules and stored at -80°C. The concentrations of Mtx, D3M, D5M, D8M and D10M were determined by Beer's law using an extinction coefficient of ε = 6700 $cm^{-1}M^{-1}$ (calculated from a known solution of Mtx in DMF) for all five compounds. Dex solutions were prepared on a sufficient scale to measure the amount of Dex accurately. The yeast two-hybrid plasmids, *pMW102*, *pMW103*, and *pMW106* were courtesy of Dr. Roger Brent. *S. cervisiae* strain FY250 was kindly provided by Dr. David McNabb.

Construction of the LexA- and B42-Protein Chimeras.

Plasmids encoding the LexA- and B42-protein chimeras were prepared using standard molecular biology techniques.[31, 32] Plasmids *pMW3eDHFR* and *pMW2Gly6rGR2* were described previously.[1]

The gene encoding the *E. coli* DHFR (eDHFR) was subcloned from *pMW3eDHFR* to *pMW102*. A 477 base pair (bp) *Mfe*I to *Xho*I fragment encoding eDHFR was prepared by PCR from pMW103-eDHFR using the primers 5'-GCA TTG CTG CAA TTG ATC AGT CTG ATT GCG GCG TTA GCG-3' (*Mfe*I, coding strand) and 5'-GCA TAC AGC CTC GAG TTA CCG CCG CTC CAG AAT CTC AAA G-3' (*Xho*I, non-coding strand). This fragment was inserted between the *Eco*RI site and the *Xho*I site in *pMW102* to give to *pMW2eDHFR*.

The gene encoding eDHFR was also subcloned from *pMW2eDHFR* to both *pMW102* and *pMW103* with a glycine-serine-glycine-glycine-serine-glycine $(GlySerGly)_2$ linker added between the fusion domains. A 495 bp *Mfe*I to *Xho*I fragment encoding $(GlySerGly)_2$-eDHFR was prepared by PCR from *pMW3eDHFR* using the primers 5'-GCA TAC GTC CAA TTG GGT TCT GGT GGT TCT GGT ATC AGT CTG ATT GCG GC-3' (*Mfe*I, coding strand) and 5'-GCA TAC AGC CTC GAG TTA CCG CCG CTC CAG AAT CTC AAA G-3' (*Xho*I, non-coding strand). This fragment was inserted between the *Eco*RI site and the *Xho*I site in *pMW102* to give *pMW2(GSG)2eDHFR* and between the *Eco*RI site and the *Xho*I site in *pMW103* to give *pMW3(GSG)2eDHFR*.

The gene encoding the murine DHFR (mDHFR) was subcloned from plasmid *pMUD2* to *pMW102* and *pMW103*. A 561 bp *Eco*RI to *Xho*I fragment encoding mDHFR was prepared by PCR from *pMUD2* using the primers 5'-GCA TAC GTC GAA TTC GTT CGA CCA TTG AAC TGC-3' (*Eco*RI, coding strand) and 5'-GCA TTG CTG CTC GAG TTA GTC TTT CTT CTC GTA GA-3' (*Xho*I, non-coding strand). This fragment was inserted between the *Eco*RI site and the *Xho*I site in *pMW102* to give *pMW2mDHFR* and between the *Eco*RI site and the *Xho*I site in *pMW103* to give *pMW3mDHFR*.

The gene encoding mDHFR was also subcloned from plasmid *pMW2mDHFR* to *pMW102* and *pMW103* with the $(GlySerGly)_2$ linker added between the fusion domains. A 579 bp *Eco*RI to *Xho*I fragment encoding $(GlySerGly)_2$-mDHFR was prepared by PCR from *pMW2mDHFR* using the primers 5'-GCA TAC GTC GAA TTC GGT TCT GGT GGT TCT GGT GTT CGA CCA TTG AAC TGC-3' (*Eco*RI, coding strand) and 5'-GCA TTG CTG CTC GAG TTA GTC TTT CTT CTC GTA GA-3' (*Xho*I, non-coding strand). This fragment was inserted between the *Eco*RI site and the *Xho*I site in *pMW102* to give *pMW2(GSG)2mDHFR* and between the *Eco*RI site and the *Xho*I site in *pMW103* to give *pMW3(GSG)2mDHFR*.

The gene encoding the hormone-binding domain (amino acids 524-795) of the rat glucocorticoid receptor with mutations F620S and C656G (rGR2) was subcloned from *pMW2Gly6rGR2* to *pMW102*. An 819 bp *Mfe*I to *Xho*I fragment encoding rGR2 was prepared by PCR from *pMW2Gly6rGR2* using the primers 5'-GCA TTG CTG CAA TTG ATC

AGT CTG ATT GCG GCG TTA GCG-3' (*Mfe*I, coding strand), 5'-GCA TAC AGC CTC GAG TTA CCG CCG CTC CAG AAT CTC AAA G-3' (*Xho*I, non-coding strand). This fragment was inserted between the *Eco*RI site and the *Xho*I site in *pMW103* to give *pMW3rGR2*.

The gene encoding rGR2 was also subcloned from *pMW2rGR2* to both *pMW102* and *pMW103* with the $(GlySerGly)_2$ linker added between the fusion domains. A 437 bp *Mfe*I to *Xho*I fragment encoding $(GlySerGly)_2$-rGR2 was prepared by PCR from *pMW2rGR2* using the primers 5'-GCA TAC GTC CAA TTG GGT TCT GGT GGT TCT GGT GCA GGA GTC TCA CAA GA-3' (*Mfe*I, coding strand) and 5'-GCA TTG CTG CTC GAG TCA TTT TTG ATG AAA CAG AAG-3' (*Xho*I, non-coding strand). This fragment was inserted between the *Eco*RI site and the *Xho*I site in *pMW102* to give *pMW2(GSG)2rGR2* and between the *Eco*RI site and the *Xho*I site in *pMW103* to give *pMW3(GSG)2rGR2*.

Construction of the yeast strains

The yeast strains described in this paper (Table I) were prepared using a lithium acetate transformation method followed by selection on synthetic complete (SC) media containing 2% glucose and lacking the appropriate selective nutrients as described.[27] Strain V248Y was prepared previously by transforming *S. cerevisiae* strain FY250 with plasmid *pMW106*, which encodes the *lacZ* gene under the control of eight tandem LexA operators. Strains containing plasmids encoding the LexA-

and B42-fusion proteins were constructed by transforming strain V248Y with the appropriate *pMW102* and *pMW103* derivatives and growing the resulting strains on the appropriate SC media.

Table I. Strains Used in This Study

Strain	Genotype
EGY40	*MATα ura3-52 trp1 his3 leu2::*0 LexA op-*LEU2 gal*$^+$
V134Y	EGY40 pPE290, pPE293, pSH18-34
FY250	*MATα ura3-52 trp1 63 his3 200 leu2 1 gal*
V248Y	FY250 pMW106
V379Y	FY250 pMW106, pMW3eDHFR, pMW102
V381Y	FY250 pMW106, pMW103, pMW102
V494Y	FY250 pMW106, pMW3eDHFR, pMW2(GSG)2rGR2
V504Y	FY250 pMW106, pMW3(GSG)2mDHFR, pMW2(GSG)2rGR2
V506Y	FY250 pMW106, pMW3(GSG)2rGR2, pMW2eDHFR
V512Y	FY250 pMW106, pMW3(GSG)2rGR2, pMW2(GSG)2mDHFR
V560Y	FY250 pMW106, pMW103, pMW2(GSG)2rGR2
V822Y	FY250 pJK101
V823Y	FY250 pJK101, pMW103
V824Y	FY250 pJK101, pMW3eDHFR
V825Y	FY250 pJK101, pMW3(GSG)2mDHFR
V826Y	FY250 pJK101, pMW3(GSG)2rGR2

Western blots

Western blots were carried out essentially as described.[32] The yeast strains were grown to mid-log phase (OD_{600} = 0.5-0.9)

in the appropriate SC media and then lysed using acid-washed glass beads. The total protein concentration for each lysed cell extract was quantified using a Bradford assay such that 2 µg of total protein was analyzed for each strain tested. Relative protein expression levels were determined by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) followed by transfer to a PVDF membrane and Western staining. The washing buffer for the Western staining was 20 mM Tris pH 7.6, 70 mM NaCl, and 0.1% Tween. The blocking buffer was the same, but with 5% non-fat dry milk. The PVDF membrane was incubated with the primary antibody at a 10,000:1 dilution (anti-LexA IgG) or a 1000:1 dilution (anti-HA IgG) for 2 hrs. The incubation with the secondary antibody was at a 1000:1 dilution for 1 hr. The secondary antibody was detected by the incubation of the membrane with 20:1 dilution of luminol and peroxide for 1 min with vigorous shaking. The membrane was visualized by exposing it to autoradiography film for 1 min.