Novel Inhibitors of Erm Methyltransferases from NMR and Parallel Synthesis

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The Erm family of methyltransferases confers resistance to the macrolide−lincosamide−streptogramin type B (MLS) antibiotics through the methylation of 23S ribosomal RNA. Upon the methylation of RNA, the MLS antibiotics lose their ability to bind to the ribosome and exhibit their antibiotic activity. Using an NMR-based screen, we identified a series of triazine-containing compounds that bind weakly to ErmAM. These initial lead compounds were optimized by the parallel synthesis of a large number of analogues, resulting in compounds which inhibit the Erm-mediated methylation of rRNA in the low micromolar range. NMR and X-ray structures of enzyme/inhibitor complexes reveal that the inhibitors bind to the *S*-adenosylmethionine binding site on the Erm protein. These compounds represent novel methyltransferase inhibitors that serve as new leads for the reversal of Erm-mediated MLS antibiotic resistance.

Introduction

A major form of resistance to the macrolide−lincosamide−streptogramin type B (MLS) antibiotics in pathogenic bacteria results from a base-specific methylation of bacterial 23S ribosomal RNA near or within the macrolide binding site.[1] As a result, the MLS antibiotics lose their ability to bind to the ribosome and no longer exhibit antibacterial activity. RNA methylation occurs through the action of the Erm (erythromycin-resistance methylase) family of methyltransferases. This family of enzymes catalyzes the mono- or dimethylation of adenine at the N6 position using *S*-adenosylmethionine (AdoMet) as the source of the methyl group.[2] Compounds which inhibit Erm methyltransferases can sensitize MLS-resistant bacteria to macrolide antibiotics as demonstrated both in vitro and in vivo for ErmC methyltransferase inhibitors.[3] These studies suggest that Erm inhibitors used in combination with a broad-spectrum macrolide antibiotic could be useful for the treatment of infections caused by MLS-resistant pathogenic bacteria.

We have previously reported on the three-dimensional structure of the ErmAM methyltransferase as determined by NMR spectroscopy.[4] The structure of the catalytic domain of ErmAM was found to be similar to that of other methyltransferases, consisting of a seven-stranded β-sheet flanked by α-helices and a small, two-stranded β-sheet.[5,6] Subsequently, crystal structures of ErmC′ have been reported which reveal the binding mode of AdoMet and AdoMet analogues to the catalytic domain.[7,8] Here we describe the discovery of novel inhibitors of the ErmAM and ErmC′ methyltransferases which bind to the AdoMet binding site and inhibit the ability of these Erm proteins to methylate rRNA.

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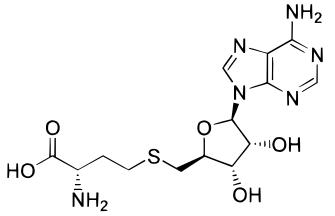
Results and Discussion

Identification and Design of Initial Lead Ligands. Using the SAR by NMR™ method,[9,10] a library of small, organic molecules was screened against ErmAM. Several classes of compounds were identified which bind to the enzyme in the millimolar range, including 2-aminobenzthiazole (**1**), 7-azaindole (**2**), and 2,4-diamino-6-(methylthio)-1,3,5-triazine (**3**) (Table 1). All of these compounds cause similar amide chemical shift changes as *S*-adenosyl-L-homocysteine (SAH, **7**) and bind competitively with this naturally occurring inhibitor of the Erm family of proteins. These results indicate that the NMR-derived leads bind to the active site of ErmAM.



7, $K_I \sim 40\ \mu M$

Analogues of these initial leads were obtained from our compound repository and tested for binding to ErmAM. 2-Amino-4-(cyclohexylamino)-6-(4-hydroxyphenyl)-1,3,5-triazine, **4**, exhibited a 3-fold improvement in binding relative to the lead triazine **3**. Removal of the cyclohexyl substituent of **4** reduced the binding affinity of the triazine by 40-fold (**5**). However, replacement of the phenol moiety of **5** by a piperidinyl group (**6**) showed an increase in binding. This observation suggested that replacement of the phenol group of **4** with a piperidinyl group should lead to an increase in binding affinity. On the basis of this hypothesis, a compound (**8**, Table 2) was prepared according to Scheme 1. This compound was found to have a K_D value of less than 0.1 mM by NMR and exhibited an IC_{50} value of 75 μM against ErmAM in an in vitro inhibition assay.

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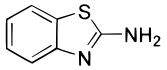
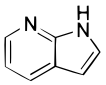
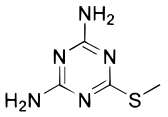
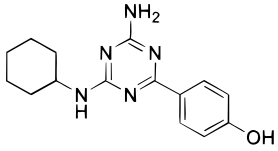
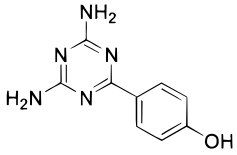
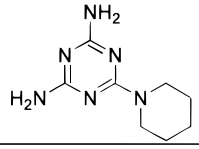
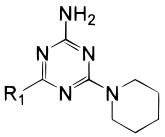
Table 1. Compounds That Bind to ErmAM Identified by NMR

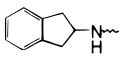
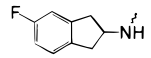
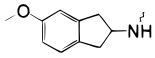
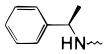
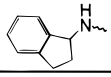
No.	Structure	NMR K_D (mM)
1		1.0
2		1.3
3		1.0
4		0.31
5		12.0
6		5.0

Optimization of the Triazine Leads. The triazine lead **8** was readily amenable to optimization using a series of solution-phase parallel synthetic approaches. A total of 232 compounds with a diverse set of substituents at R_1 were synthesized according to Scheme 1 and tested for their ability to inhibit ErmAM and ErmC′. A subset of these compounds along with their inhibitory constants (K_I) for ErmAM and ErmC′ are shown in Table 2. The incorporation of 2-aminoindan (**9**) resulted in an order of magnitude increase in inhibitory potency against ErmAM relative to **8**. Compounds containing analogues of 2-aminoindan (e.g., **10**, **11**) also inhibited ErmAM and ErmC′ with potencies similar to that of **9**. However, compounds containing other hydrophobic substituents (e.g., **12**−**15**) at this position or even a regioisomer of 2-aminoindan (e.g., **16**) were significantly less active, suggesting that the 2-aminoindan binding site is very specific.

A total of 411 compounds were synthesized according to Scheme 1 to explore the SAR at R_2 (Table 3). No substantial gains in potency were observed for piperidinyl analogues, as illustrated for a few compounds that exhibited comparable ErmAM potencies (e.g., **17**−**19**). Although negatively charged substituents (e.g., **20**), cyclohexylamine (**21**), and other substitutions at R_2 resulted in significant decreases in activity, most of the substitutions at this position gave rise to similar ErmAM potencies (e.g., **22**−**26**). The best inhibitor against ErmC′ contained a *m*-aniline (**26**) and was synthesized according to Scheme 2.

Table 2. ErmAM and ErmC′ Methylase Inhibition Activities for Substituted 2-Amino-4-piperidinyl-1,3,5-triazines



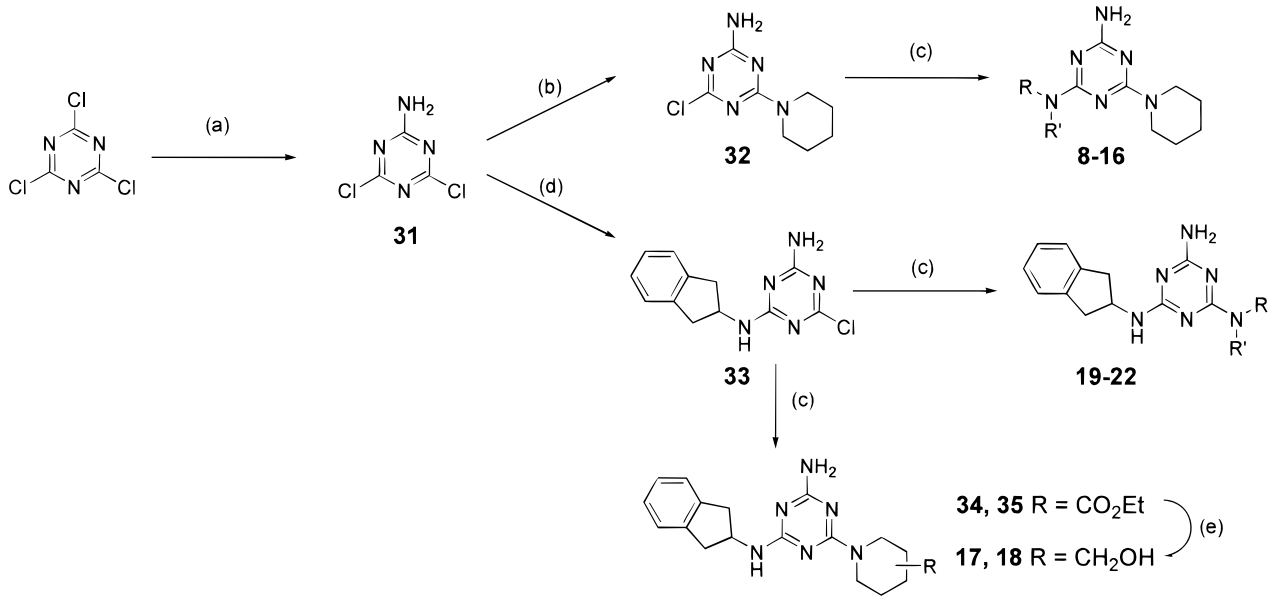
No.	R_1	Erm-AM K_I (μM)	ErmC' K_I (μM)
8		75	>100
9		8	75
10		3	37
11		6	75
12		>100	>100
13		>100	>100
14		>100	>100
15		>100	>100
16		75	>100

A few compounds were also prepared to examine the importance of the exocyclic amine at R_3 and the triazine core (Table 4). Compounds lacking the amine (**27**, Scheme 3) or those with a hydroxyl substituent (**28**, Scheme 3) exhibited a significant decrease in their ability to inhibit ErmAM. Incorporation of a pyrimidine core (**29**) led to a 2-fold decrease in potency against ErmAM relative to **25**; however, modifications of the *p*-aniline group (**30**) resulted in substantial gains in activity against ErmAM and ErmC′.

Structural Studies of Lead Compounds. To provide a structural basis for the inhibition of Erm methyltransferases by the lead compounds, NMR and X-ray crystal structures of Erm/ligand complexes were obtained. An NMR-derived model of the complex between ErmAM and **9** was obtained based on 18 intermolecular NOEs (Figure 1). Consistent with the NMR binding data, **9** was found to bind to the active site of ErmAM. The triazine moiety of **9** overlays well with the adenine of SAH and, like SAH, is able to form hydrogen bonds with the backbone NH of I84 and the side chain carboxylate of D83. In addition, the exocyclic amine at R_3 is able to form a hydrogen bond with the backbone carbonyl of I57 of ErmAM. This hydrogen bond is absent in SAH due to the lack of an analogous exocyclic amino substituent on the adenine ring (see Figure 1). The importance of this hydrogen bond in the binding of the triazine inhibitors to ErmAM and ErmC′ is consistent with the significant decreases in activity observed upon the removal of the amine (**27**) or when it was replaced with a hydroxyl group (**28**). The 2-aminoindan substituent makes NOE contacts with residues L85, L105, Q108, and I109 in ErmAM. This hydrophobic pocket is also not utilized by SAH (Figure 1).

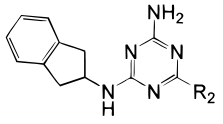
In addition to the NMR structure, a 3.0 Å X-ray crystal structure of ErmC′ complexed with **30** was obtained (Figure 2). Consistent with the NMR-derived

Scheme 1. Synthesis of Substituted Triamino-1,3,5-triazines[a]



[a] Reagents and conditions: (a) ammonia gas, ether, −10 °C, 1 h; (b) piperidine, ice, 0 °C to room temperature, 3.5 h; (c) HNRR′, H_2O, reflux, 4 h; (d) 2-aminoindan, ice, 0 °C to room temperature, 3.5 h; (e) BH_3·THF.

Table 3. ErmAM and ErmC′ Methylase Inhibition Activities for Substituted 2-Amino-4-(aminoindanyl)-1,3,5-triazines



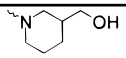
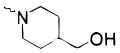
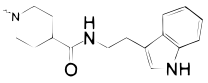
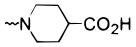
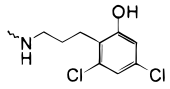
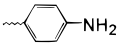
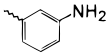
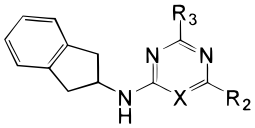
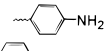
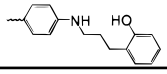
No.	R_2	Erm-AM K_I (μM)	ErmC' K_I (μM)
17		12.5	>100
18		9	75
19		2	20
20		>100	>100
21		>100	>100
22		10	>100
23	SCH_3	10	>100
24		25	>100
25		35	>50
26		4	10

Table 4. ErmAM and ErmC′ Methylase Inhibition Activities for Aminoindanyl-Substituted 1,3,5-Triazines and Pyrimidines

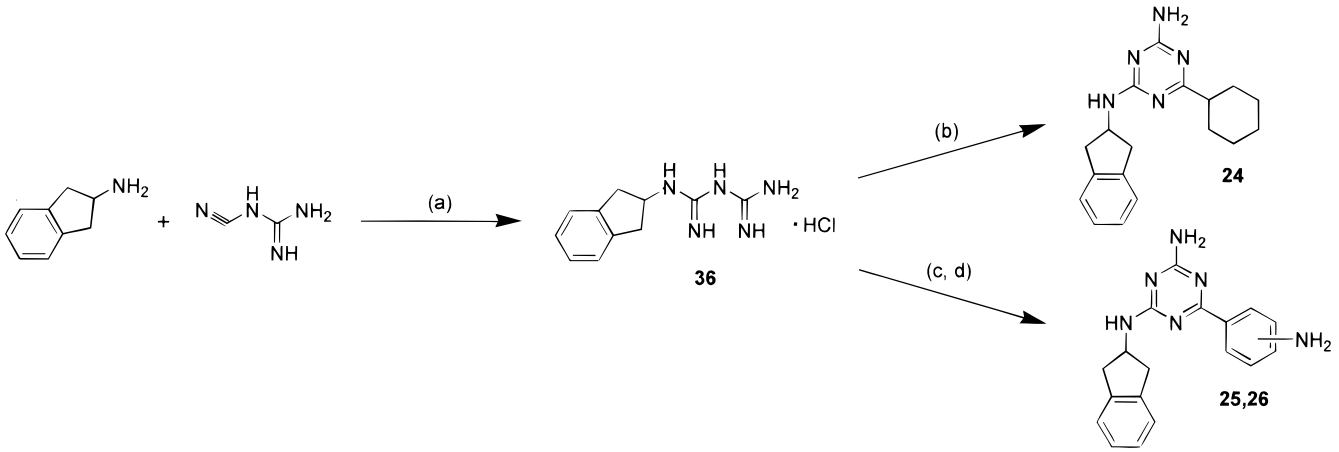


No.	R_2	R_3	X	Erm-AM K_I (μM)	ErmC' K_I (μM)
27		H	N	>75	>100
28		OH	N	>100	>100
29		NH_2	C	75	>100
30		NH_2	C	7.5	12.5

model of ErmAM complexed with **9**, the aminopyrimidine core of **30** occupies the same site as the adenine moiety of SAH, and the exocyclic amine at R_3 was found to donate a hydrogen bond to I58. However, unlike SAH, the distance between the endocyclic N1 of the pyrimidine (analogous to N1 in adenine) and the backbone amide of I85 is too long for a strong hydrogen bond (4.4 Å), even though they are geometrically aligned. The orientation of the 2-aminoindan of **30** is consistent with the observed NOEs between compound **9** and ErmAM. The indan group was found to fit into a hydrophobic crevice composed of the side chains of I85, L86, and I106 and the hydrophobic portion of the side chains of D84 and D109. The bridging exocyclic amine at R_1 was found to be pointing away from D84, facilitating a close fit between the indan group and the hydrophobic exosite.

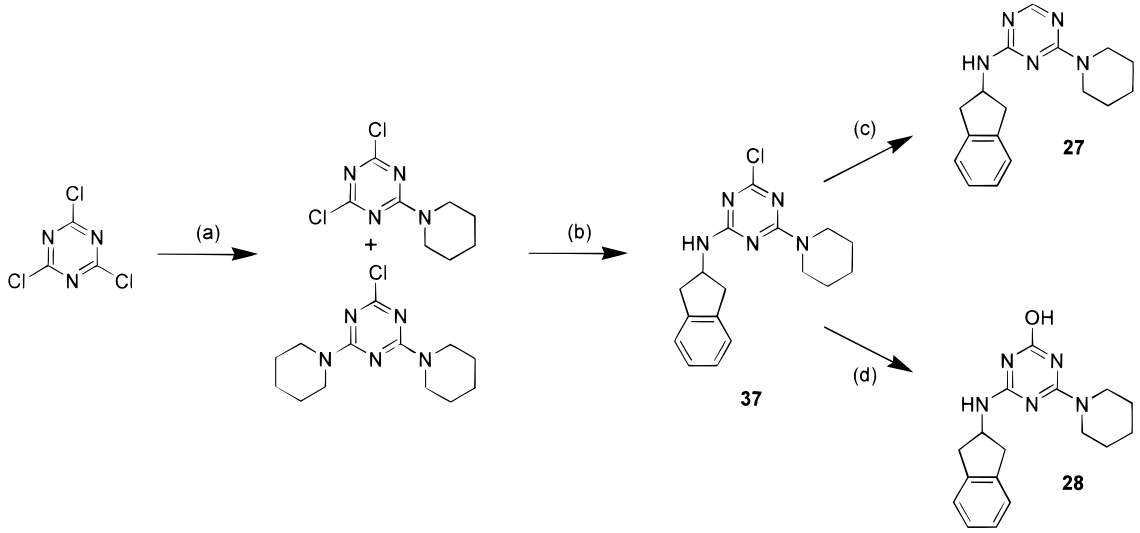
The specific interactions observed for the complexes of ErmAM and **9** and ErmC′ and **30** are very similar. Both structures indicate hydrogen bonding with the triazine moiety and extensive hydrophobic interactions with the indan substituent. The active sites in ErmAM and ErmC′ are highly homologous, and of the residues shown to interact directly with the ligands, the only differences are for I106 (L105) and D109 (Q108) in ErmC′ (ErmAM). The change from aspartic acid to glutamine may explain some of the potency differences for the compounds between ErmAM and ErmC′, since the interaction with these residues involves the hydrophobic portion of the side chain. Additional differences in sequence between ErmAM and ErmC′ that may affect ligand binding are for residues I60 (L59) and S39 (T38) in ErmC′ (ErmAM), which can potentially interact with the planar face of the triazine ring and with substituents at R_2, respectively.

Scheme 2. Synthesis of Substituted Diamino-1,3,5-triazines[a]



[a] Reagents and conditions: (a) neat, 150−160 °C, 1 h; (b) cyclohexanecarbonyl chloride, aq NaOH; (c) 4-nitrobenzoate/3-nitrobenzoate, NaOMe−MeOH/reflux; (d) H_2/5% Pd/$BaSO_4$, EtOH−THF/room temperature.

Scheme 3. Synthesis of Substituted 4-(Piperidinyl)-6-(2′-aminoindanyl)-1,3,5-triazines[a]



[a] Reagents and conditions: (a) piperidine, −45 °C; (b) aminoindan, Et_3N; (c) Pd/C, H_2; (d) 6 N HCl, reflux.

In both the NMR and X-ray structures, the substituents at R_2 (piperidine of **9** and aniline of **30**) only partially fill the space occupied by the ribose ring of SAH, and the site of interaction for the amino acid moiety of SAH is completely unoccupied. In fact, in the X-ray structure, the anilino moiety at R_2 was not well-ordered in any electron density map, and the aminopropylphenol portion of the inhibitor was found to orient toward the surface of the cofactor binding site rather than back into the pocket occupied by the amino acid portion of SAH. This most likely accounts for the lack of specificity at R_2, but it also suggests that further gains in potency could be achieved by properly occupying these binding sites.

Concluding Remarks

The ability to identify lead compounds that can be used as starting points in the design of high-affinity ligands is a critical aspect of drug discovery. Using conventional high-throughput screening of large libraries of compounds, small, potent molecules that can be readily modified cannot always be found. SAR by NMR offers an attractive alternative approach for the discovery and design of novel leads which do not exist in corporate repositories. In fact, using this NMR-based approach, leads for the HPV-E2 protein[11] and the matrix metalloproteinase stromelysin[12] were discovered when traditional high-throughput screening failed to produce leads. Here we have described how novel Erm inhibitors were discovered using NMR-based screening coupled with parallel synthesis. These Erm inhibitors are small and amenable to a wide variety of chemical modifications. In addition, based on the NMR and X-ray structures of these compounds complexed to ErmAM and ErmC′, there is ample room for modification to take advantage of additional interactions in the active site. Significantly, these compounds are nonnucleoside in nature and thus may serve as selective inhibitors of the Erm methyltransferases, circumventing the selectivity problems which may be encountered using compounds derived from SAH, a nonselective methyltransferase inhibitor. In fact, preliminary data on the initial triazine leads indicate that they exhibit little to no inhibitory effect on *Eco*RI DNA methylase activity. Thus, these Erm inhibitors serve as useful leads in the search for

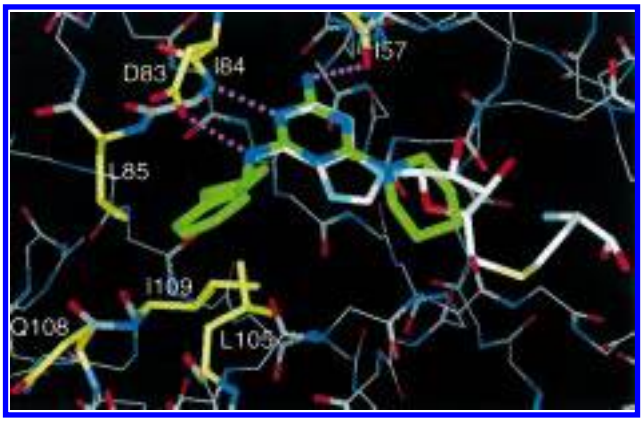


Figure 1. NMR-derived model of the complex between ErmAM (gray ribbon) and **9** (green carbon atoms). The binding orientation of SAH (white carbon atoms) based on the crystal structure of ErmC′ [6] is also shown. Hydrogen bonds between **9** and ErmAM are shown as dotted lines. Residues which show NOEs to **9** (white residue labels) and those which are involved in hydrogen bonds (yellow residue labels) are shown with yellow carbon atoms.

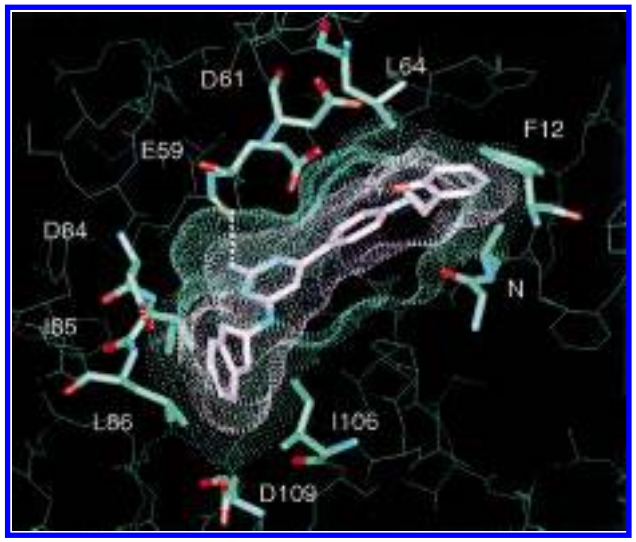


Figure 2. Crystal structure of ErmC′ complexed with **30**. Shown in green are the amino acid residues involved in contacts with the inhibitor shown in pink. Also shown is the contact surface between the two molecules as calculated by the program package QUANTA97. The hydrogen bond between the inhibitor and the protein is indicated by the dashed white line.

therapeutics which reverse Erm-mediated MLS antibiotic resistance.

Experimental Section

General. All melting points were determined using a Hoover capillary melting point apparatus and are uncorrected. Reagents and starting materials were obtained from commercial suppliers and used without further purification unless otherwise indicated. All reaction solvents were purchased in anhydrous form and not dried further. Flash column chromatography was carried out on silica gel (230−400 mesh). HPLC grade solvents were used for all chromatography. Analytical thin-layer chromatography (TLC) was conducted on precoated plates: silica gel 60 F-254, 0.25-mm thickness, manufactured by E. Merck & Co., Germany. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Bruker AM-300 (300 MHz) spectrometer in DMSO-d_6 unless otherwise indicated. Low-resolution mass spectra were determined on a Finnigan-Matt SSQ700 mass spectrometer. High-resolution mass spectra were determined on a JEOL JMS-SX102A-hybrid mass spectrometer. Reactions at 0 °C were carried out in an ice/water bath. Reactions at −78 °C were carried out in a dry ice/acetone bath. The term concentrated in vacuo refers to the removal of volatile materials on a rotary evaporator at reduced pressure (30−80 Torr).

Detection of Ligand Binding Using NMR. ErmAM was expressed and purified as previously described.[4] NMR samples were composed of uniformly ^{15}N-labeled ErmAM at 0.3 mM in a H_2O/D_2O (9:1) solution containing 20 mM BisTris, 10 mM DTT, pH 6.5. Ligand binding was detected at 30 °C by acquiring sensitivity-enhanced ^{15}N HSQC spectra[13] on 500 μL of ErmAM in the presence and absence of added compound. Compounds were added as solutions in perdeuterated DMSO. A Bruker sample changer was used on a Bruker AMX500 spectrometer. Compounds were initially tested at 1.0 mM each, and binding was determined by monitoring changes in the ^{15}N HSQC spectra. Dissociation constants were obtained for selected compounds by monitoring the chemical shift changes as a function of ligand concentration. Data were fit using a single-binding site model. A least-squares grid search was performed by varying the values of K_D and the chemical shift of the fully saturated protein.

NMR Structural Studies. Experiments were performed at 30 °C on a Bruker AMX600 spectrometer. NMR samples were composed of uniformly ^{15}N/^{13}C-labeled ErmAM at 1.0 mM in a D_2O/ H_2O (9:1) solution containing 1.0 mM **9**, 20 mM phosphate, 1 mM perdeuterated DTT, pH 6.5 (uncorrected). Backbone and side chain assignments of ErmAM used in solving the structure[4] were used to assign the signals of the complex. This was accomplished by comparing the peak patterns in the NMR spectra of ErmAM in the presence and absence of added compound. NOEs between **9** and ErmAM were obtained from ^{13}C-edited NOESY−HMQC[14] and 3D ^{13}C-edited/^{12}C-filtered HSQC−NOESY spectra[14,15] with a mixing time of 80 ms. A total of 18 intermolecular NOEs between the ligand and the protein were unambiguously assigned. A model of the complex was obtained by manually docking **9** onto the structure of ErmAM derived from the crystal structure of ErmC′ complexed with SAH.[8]

X-ray Crystal Studies. ErmC′ was prepared for crystallization as described.[8] Crystals of apo-ErmC′ were grown by the hanging drop vapor diffusion method with some modification. Specifically, drops containing 5−8 mg/mL ErmC′ in 25 mM Tris/Cl, 100 mM NaCl, 2 mM DTT, 10% (v/v) glycerol, pH 7.5, were equilibrated against a reservoir containing 100 mM Tris, 500 mM $NH_4(SO)_4$, 15% PEG 8000, pH 7.8. Crystals appeared within 1 day and grew to full size within 1 week. Crystals belonged to the space group $P4_32_12$, $a = b = 81.5$ Å, $c = 121.96$ Å, $\alpha = \beta = \gamma = 90°$, and diffracted to a resolution of 3.0 Å. Compound **30** was dissolved in 100% DMSO at a concentration of 100 mg/mL. A single ErmC crystal was placed in 50 μL of 20% PEG 8000, 0.3 M ammonium sulfate, 10% glycerol, pH 7.7, and 0.5 μL of the compound solution was added. Crystals were allowed to equilibrate for 4 h. Data were collected on a Rigaku RTP 300 RC rotating anode source equipped with a MAR CCD detector at 100 K by the method of flash-freezing. Data consisted of 12 2.0° oscillations which were each exposed for 15 min and were processed using the HKL program.[16] Initial electron density maps were calculated using the program package XPLOR[17] and the native model of ErmC′.[8] All electron density maps were inspected on a Silicon Graphics INDIGO2 workstation using QUANTA 97, and the orientation of compound **30** was clearly visualized in the initial $2F_o-F_c$ map. Refinement utilized the program REFMAC[18] in the CCP4 package.[19] Protein restraints were defined by the package PROTIN and the inhibitor restraints were defined by MAKEDICT as implemented in CCP4 using a model generated with the drawing feature and minimized with the CHARMM module of QUANTA97. Refinement utilized data greater than 1.5σ above background and also included restrained *B*-factor refinement as implemented in REFMAC. The initial $2F_o-F_c$ electron density map for **30** showed density for all atoms of the inhibitor, although that for the phenyl at R_2 was much weaker than that for the rest of the molecule. The N-terminus

of the ErmC′ structure was partially disordered and begins with the main chain of residues 8 and 9. The side chains of these amino acids were disordered in the final model. The final model was refined to a crystallographic *R*-factor of 19.5% (free *R*-factor = 27.0%) for data up to 3.0 Å and contains 15 solvent molecules defined as peaks greater than 4.0σ in the F_o-F_c map.

Inhibition Assays. ErmAM (ErmC′) inhibitory activity was determined in a reaction mixture consisting of 50 mM Tris, pH 7.5, 100 (40) mM KCl, 4 mM $MgCl_2$, 1 (10) mM DTT, and 95 (143) nM ErmAM (ErmC′). Test compound (1−4 μL) was added in duplicate to 96-well v-bottom polypropylene plates (Costar), to which 24 μL of the reaction mixture containing the enzyme was added. This mixture was gently shaken for 15 min at room temperature, at which time 25 μL of a solution containing 250 nM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (Amersham Pharmacia Biotech) and 80 nM *E. coli* rRNA (Boehringer-Mannheim) was added. After gently shaking for an additional 2 h at room temperature,[20] the reaction was terminated by adding 150 μL of a 10% TCA solution containing 100 mM KCl to each well and cooling on ice for 30 min. The samples were filtered using GF/B 96-well filter plates (Millipore) presoaked with 75 μL of a solution containing 50 mM Tris, pH 7.5, 100 mM KCl, and 100 μg/μL BSA. Wells were rinsed five times with 200 μL of a 5% TCA solution, with a final wash of 200 μL of 95% cold ethanol. Filters were dried for 45 min at 310 K. TCA-precipitated counts were quantitated in a Packard Top Count after the addition of 50 μL of MicroScint-O (Packard). IC_{50} values were obtained by graphing percent inhibition, relative to control wells without inhibitor, as a function of inhibitor concentration. The K_I value is given by $K_I = IC_{50}/(1 + [AdoMet]/K_M)$, with the K_M for AdoMet equal to 300 nM under the conditions of the assay. The reported K_I values are derived from this equation with an estimated error in the K_I value of ±50%.

2-Amino-4-chloro-6-(2-aminoindanyl)-1,3,5-triazine (33). Powdered 2-amino-4,6-dichloro-1,3,5-triazine (**31**)[21,22] (3.4 g, 20.9 mmol) was added to crushed ice (100 g) and 2-aminoindan (5.4 mL, 41.7 mmol) was added in a single step with stirring. The mixture became thick almost immediately. Stirring was continued for 3.5 h while the reaction was allowed to warm to room temperature. The precipitate was filtered off and was recrystallized from EtOAc to afford 3.2 g of the product as pale gray crystals: 58% yield; ^{1}H NMR (DMSO-d_6) δ 7.64 (1H, bs), 7.11−7.19 (4H, cm), 6.89 (2H, bs), 4.58−4.63 (1H, cm), 3.19−3.24 (2H, dd, J = 4.9, 15.9 Hz), 2.86−2.90 (2H, dd, J = 7.0, 15.9 Hz). Anal. ($C_{12}H_{12}N_5Cl$) C, H, N. FAB HRMS: MH^+ calcd for $C_{12}H_{13}N_5Cl$, 262.086; found, 262.086.

General Method For the Solution-Phase Parallel Synthesis of the Triaminotriazines 8−16, 19−21, 34, and 35.[21] The reactions were carried out in 25-mL screw cap vials in batches of 25 reactions at a time. A representative procedure is as follows for the conversion of **32**:[22] A mixture of 2-amino-4-chloro-6-(piperidin-1-yl)-1,3,5-triazine (50 mg, 0.23 mmol) and the appropriate amine (5 equiv) in H_2O (1.0 mL) was heated to 100 °C for 4 h. After cooling, 2 N aq NaOH (0.12 mL, 0.23 mmol) and 3.0 mL of H_2O were added. The solution was then extracted in the vial with 15.0 mL of ether. The organic layer was decanted and evaporated in a speed vac and the crude product was purified by preparative reverse-phase HPLC. MS and analytical reverse-phase HPLC showed that the products were obtained in purities >80%.

2-Amino-4-(3-hydroxymethylpiperadino)-6-(2-aminoindanyl)-1,3,5-triazine (17). A THF solution of DIBAL-H (0.8 mL, 0.8 mmol) was added dropwise to a cool (0 °C) solution of **34** (100 mg, 0.26 mmol) in THF (1 mL). Ethyl acetate (1 mL) was added after 30 min followed by 1 N HCl (5 mL). The acidic solution was neutralized with a saturated solution of $NaHCO_3$ until pH ∼ 6. The aqueous phase was extracted with EtOAc (3 × 50 mL). The organics were then combined, washed with H_2O (2 × 50 mL) and brine (25 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by flash chromatography (EtOAc) to afford 87 mg of pure alcohol: 95% yield; ^{1}H NMR (DMSO-d_6) δ 7.15 (4H, m), 6.78 (1H, d, J = 7.1 Hz), 6.1 (2H, m), 4.62 (2H, m), 4.46 (2H, t, J = 5.4 Hz), 3.22 (4H, m), 2.83 (2H, dd, J = 7.8, 15.9 Hz), 2.75 (2H, m), 1.8−1.1 (5H, series of mult.); MS (M + H) 341. Anal. ($C_{18}H_{24}N_6O$) Theory: C 63.51, H 7.11, N 24.69. Found: C 63.83, H 7.49, N 22.48.

2-Amino-4-(4-hydroxymethylpiperadino)-6-(2-aminoindanyl)-1,3,5-triazine (18). In a fashion similar to **17**, **18** was prepared using 230 mg of **35** as the starting material. All other reaction conditions are as listed for **17** to give 0.19 g of pure alcohol: 93% yield; ^{1}H NMR (DMSO-d_6) δ 7.18 (4H, m), 6.85 (1H, m), 6.15 (2H, m), 4.64 (3H, m), 4.46 (1H, t, J = 5.1 Hz), 3.24 (2H, t, J = 5.1 Hz), 3.17 (2H, m), 2.83 (2H, dd, J = 7.35, 15.81 Hz), 2.68 (2H, br t, J = 12.1), 1.60 (3H, m), 1.0 (2H, m); MS (M + H) 341. Anal. ($C_{18}H_{24}N_6O$·[0.15 CH_2Cl_2, 0.15EtOAc]) Theory: C 61.47, H 7.02, N 22.94. Found: C 61.50, H 7.05, N 22.94.

2-Amino-4-(*N*-2,4-dichloro-6-hydroxy-2-propylaminophenyl)-6-(2-aminoindanyl)-1,3,5-triazine (22). A solution of amino-3-(2,4-dichloro-6-hydroxyphenyl)propyl benzyl ether (115 mg, 0.37 mmol) and amino-3-(*N*-2-aminoindanyl)-5-chlorotriazine (97 mg, 0.37 mmol) in 6 mL of THF was heated to reflux for 16 h. TLC (75% EtOAc/hex) showed the insertion to be complete. The reaction was evaporated and purified by flash chromatography using 75% EtOAc/hex to give 164 mg (83%) of the benzyl-protected insertion product. The benzyl ether (164 mg, 0.31 mmol) was dissolved in a mixture of 10 mL of EtOAc and 5 mL of MeOH, 40 mg of 10% palladium on carbon was added, the reaction vessel was fitted with a hydrogen balloon, and the hydrogenation proceeded at 1 atm and 25 °C and was complete after 1.5 h (TLC 2.5% MeOH/EtOAc w/0.25% NH_4OH). The reaction was filtered through Celite and concentrated to give **22** as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24−7.08 (4H, m), 6.94 (1H, d, J = 1.84 Hz), 6.83 (1H, d, J = 1.84 Hz), 5.2 (2H, bs), 4.8 (2H, m), 3.4−3.2 (4H, m), 2.88−2.81 (5H, m), 1.89 (2H, bs); MS (M + H) 446.

2-Amino-4-(thiomethyl)-6-(2-aminoindanyl)-1,3,5-triazine (23). To a solution of **33** (50 mg, 0.19 mmol) in THF (3 mL) was added sodium thiomethoxide (20 mg, 0.29 mmol) and the resulting solution was stirred at 50 °C overnight. The reaction was cooled and concentrated in vacuo. The residue was purified by flash chromatography (100:1 CH_2Cl_2:MeOH) to give 39 mg of a white powder: 75% yield; ^{1}H NMR (DMSO-d_6) δ mixture of rotamers 7.56 and 7.47 (1H, 2d, J = 7.0 Hz), 7.22−7.11 (4H, m), 6.83 (1H, bs), 6.65 (1H, bs), 4.69−4.55 (1H, m), 3.18 (2H, dd, J = 15.8, 7.4 Hz), 2.84 (2H, dd and m, J = 15.8, 7.0 Hz), 2.37 and 2.35 (3H, 2s); MS (M + H) 274. Anal. ($C_{13}H_{15}N_5S$) Theory: C 56.87, H 5.60, N 25.28. Found: C 57.12, H 5.53, N 25.62.

2-Indanylbiguanide (36). A mixture of 2-aminoindan·HCl (6.0 g, 35 mM) and dicyandiamide (3.0 g, 35 mM) was heated to 160 °C for 100 min. The reaction mixture initially melts, then resolidifies. The reaction was cooled to room temperature. The solid was recrystallized from EtOH/Et_2O (4:3) to give 5.2 g of beige crystals: 58% yield; ^{1}H NMR (DMSO-d_6) δ 7.65 (1H, bs), 7.25−7.13 (4H, m), 7.03−6.62 (5H, m), 4.41−4.34 (1H, m), 3.24−3.16 (2H, m), 2.86−2.79 (2H, m); MS (M + H) 218.

2-Amino-4-cyclohexyl-6-(2-aminoindanyl)-1,3,5-triazine (24). 2-Indanylbiguanide (**36**; 0.23 g, 0.90 mmol) was suspended in 2 N NaOH (2 mL) and cooled in an ice bath. A solution of cyclohexanecarbonyl chloride (0.12 mL, 0.90 mmol) in acetone (2 mL) was added and the resulting solution was allowed to warm to room temperature overnight. The acetone was removed in vacuo and the resulting precipitate was filtered and purified by flash chromatography (50:1 CH_2Cl_2:MeOH) to give 29 mg of a clear colorless oil: 10% yield; ^{1}H NMR (DMSO-d_6, 90 °C) δ 7.18 (2H, dd, J = 3.3, 2.4 Hz), 7.11 (2H, dd, J = 3.3, 1.8 Hz), 6.89 (1H, bs), 6.19 (2H, bs), 4.63 (1H, sextet, J = 4.5 Hz), 3.19 (2H, dd, J = 9.6, 4.5 Hz), 2.87 (2H, dd, J = 9.6, 4.5 Hz), 2.28 (1H, tt, J = 6.9, 2.4 Hz), 1.82 (2H, dd, J = 8.1, 1.5 Hz), 1.73 (2H, dt, J = 7.5, 2.1 Hz), 1.66−1.62 (1H, m), 1.48 (2H, dq, J = 7.8, 2.1 Hz), 1.33−1.14 (3H, m); MS (M + H) 310.

2-Amino-4-(4-anilino)-6-(2-aminoindanyl)-1,3,5-triazine (25). 2-Indanylbiguanide (**36**) was combined with 0.95 equiv of *p*-nitrobenzoate (0.219 g, 1.12 mmole) and the resulting mixture treated with a 0.5 M solution of NaOMe in methanol (5.7 mL, 2.83 mmol). The resulting solution was refluxed under nitrogen overnight. The reaction mixture was concentrated in vacuo and the residue suspended in water. The product (0.20 g, 51%) was collected by vacuum filtration, water-washed, dried in vacuo at room temperature, and used as isolated. The intermediate was dissolved in a mixture of EtOH (10 mL) and THF (8 mL). To this solution was added 5% Pd/$BaSO_4$ (50 mg). The resulting mixture was vacuum-degassed (3×) then exposed to a hydrogen atmosphere (balloon) at room temperature. After 6 h the reaction mixture was filtered through a pad of Celite and the filtrate concentrated in vacuo. The residue was purified by flash chromatography on silica gel (ethyl acetate−hexane) to give 0.1 g of a yellow foam: 55% yield; ^{1}H NMR (DMSO-d_6) δ 8.0 (2H, d, J = 7.9 Hz), 7.18−7.20 (2H, cm), 7.11−7.13 (2H, cm), 6.83 (1H, bs), 6.58 (2H, d, J = 7.9 Hz), 6.15 (2H, bs), 5.26 (2H, bs), 4.75 (1H, cm), 3.23−3.27 (2H, dd, J = 7.9, 15.9 Hz), 2.90−2.95 (2H, dd, J = 7.3, 15.9 Hz); MS (M + H) 318. Anal. ($C_{18}H_{18}N_6$) Theory: C 67.89, H 5.71, N 26.40. Found: C 67.52, H 5.74, N 25.85.

2-Amino-4-(3-anilino)-6-(2-aminoindanyl)-1,3,5-triazine (26). In a fashion similar to **25**, **26** was prepared using *m*-nitrobenzoate in the preparation of the intermediate. All other reaction conditions are as listed for the preparation of **25** to give 0.13 g of a yellow foam: 45% yield; ^{1}H NMR (DMSO-d_6) δ 7.52 (1H, s), 7.47 (1H, d, J = 7.3 Hz), 7.19−7.21 (2H, cm), 7.11−7.14 (2H, cm), 7.04−7.07 (1H, t), 7.0 (1H, bs), 6.7 (1H, d, J = 7.9 Hz), 6.31 (2H, bs), 4.82 (2H, bs), 4.77−4.82 (1H, cm), 3.24−3.29 (2H, dd, J = 7.6, 15.3 Hz), 2.91−2.96 (2H, dd, J = 7.3, 15.3 Hz); MS (M + H) 319. Anal. ($C_{18}H_{18}N_6$·0.25H_2O) Theory: C 66.94, H 5.79, N 26.03. Found: C 66.75, H 5.79, N 25.30.

2-Chloro-4-(*N*-piperidinyl)-6-(2-aminoindanyl)-1,3,5-triazine (37). A solution of cyanuric chloride (2.5 g, 14 mM) in diethyl ether (75 mL) was cooled to −45 °C in a dry ice/acetone bath. Piperidine (2.7 mL, 27 mM) was added and the reaction stirred for 30 min. Cold water (75 mL) was then added and the reaction was removed from the dry ice bath and allowed to warm to room temperature. The ether layer was separated, reduced to 1/3 volume in vacuo, and cooled in a dry ice/acetone bath. The precipitate was filtered to give 0.5 g of disubstituted material. The filtrate was concentrated in vacuo to give the crude product which was contaminated with disubstituted material (TLC in 3:1 hexanes/EtOAc; product R_f = 0.7, disubstituted material R_f = 0.8). The crude product was used as is in the next step.

To a solution of the crude intermediate (600 mg) in THF (10 mL) were added 2-aminoindan (230 mg, 1.7 mM; made from the HCl salt by partitioning between EtOAc and 1 N NaOH) and triethylamine (0.24 mL, 1.7 mM). The reaction was stirred overnight at room temperature. The reaction was concentrated in vacuo and the residue purified by flash chromatography (preabsorb onto silica with CH_2Cl_2; elute with 20:1 hexanes:EtOAc, then 10:1 hexanes:EtOAc) to give 170 mg of a white powder: 17% 2-step yield; mp = 180−182 °C; ^{1}H NMR (DMSO-d_6) δ 7.24−7.16 (4H, m), 5.39−5.28 (1H, m), 4.88−4.74 (1H, m), 3.78−3.63 (4H, m), 3.38−3.31 (2H, m), 2.88−2.81 (2H, m), 1.71−1.53 (6H, m); MS (M + H) 310.

4-Piperidinyl-6-(2-aminoindanyl)-1,3,5-triazine (27). A solution of **37** (80 mg, 0.24 mM) in MeOH (50 mL) was treated with 10% Pd/C (50 wt % in water, 80 mg) under 1 atm of hydrogen for 3 h at room temperature. The reaction was filtered and concentrated in vacuo and the residue purified by flash chromatography (CH_2Cl_2, then 20:1 CH_2Cl_2:MeOH) to give 40 mg of a white powder: 56% yield; mp = 175 °C (shrink), 200−205 °C (dec); ^{1}H NMR (DMSO-d_6) δ mixture of rotamers 8.50 (1H, bs), 8.24 (1H, s), 7.27−7.14 (4H, m), 4.72−4.61 (1H, m), 3.82−3.71 (4H, m), 3.26 (2H, dd overlapping m, J = 15.9, 7.5 Hz), 2.92 (2H, dd overlapping m, J = 15.9, 5.9 Hz), 1.67−1.51 (6H, m); MS (M + H) 296.

2-Hydroxy-4-piperidinyl-6-(2-aminoindanyl)-1,3,5-triazine (28). To a solution of **37** (60 mg, 0.18 mM) in THF (10 mL) was added 6 N HCl (3 mL) and the resulting solution was refluxed overnight. The reaction was cooled, basified with 6 N NaOH, and extracted with CH_2Cl_2. The organic layer was dried and concentrated in vacuo and the resifue purified by flash chromatography (CH_2Cl_2, then 10:1 CH_2Cl_2:MeOH) to give 15 mg of a white powder: 27% yield; mp = 176−180 °C; ^{1}H NMR (DMSO-d_6) δ 9.49 (1H, bs), 7.27−7.13 (4H, m), 7.02 (1H, bs), 4.63−4.57 (1H, m), 3.72−3.62 (4H, m), 3.27−3.19 (2H, m), 2.88−2.79 (2H, m), 1.51−1.43 (4H, m); MS (M + H) 312.

2-Amino-4-(4-anilino)-6-(2-aminoindanyl)pyrimidine (29). 2-Amino-1,6-dihydro-4-(4′-nitrophenyl)-6-oxopyrimidine was prepared from ethyl 4-nitrobenzoyl acetate (4.74 g, 20 mol).[23] A suspension of the crude product (2.96 g, 12.76 mmol) in $POCl_3$ (30 mL) was heated under nitrogen in an oil bath to 125−130 °C. After 3 h the mixture was cooled and the excess $POCl_3$ distilled off at 50 °C. The resulting paste was dissolved by cautious addition of ice water and the slurry made neutral/slightly basic with solid NaOH. The product was collected by vacuum filtration and water-washed. The cake was dried in vacuo at room temperature and was sufficiently pure for use as isolated in the next step.

The intermediate (0.54 g, 2.16 mmol) was combined with 2-aminoindan hydrochloride (0.37 g, 2.16 mmol) in *n*-BuOH (10 mL) containing triethylamine (0.66 mL, 4.75 mmol). The mixture was heated under nitrogen in an oil bath to 120 °C. After 15 h the reaction mixture was cooled and the solvent distilled off in vacuo at 45 °C. The residue was chromatographed on silica gel (ethyl acetate−hexane) and dissolved in a mixture of ethanol (15 mL) and THF (10 mL). To this solution was added 5% Pd/$BaSO_4$ and the resulting mixture vacuum degassed (3×) then exposed to a hydrogen atmosphere (balloon) at room temperature. After 2.5 h the reaction mixture was filtered through Celite and the filtrate concentrated in vacuo: 36% yield; ^{1}H NMR (DMSO-d_6) 7.63 (2H, d, J = 8.5 Hz), 7.19−7.21 (2H, cm), 7.11−7.12 (2H, cm), 6.59 (2H, d, J = 8.5 Hz), 6.12 (1H, s), 5.44 (2H, bs), 5.07 (2H, bs), 4.65−4.69 (1H, cm), 3.25−3.30 (2H, dd, J = 7.3, 15.9 Hz), 2.82−2.86 (2H, dd, J = 6.4, 15.9 Hz); MS (M + H) 318.

2-Amino-4-(4-[*N*-2-hydroxypropylaminophenyl]phenyl)-6-(2-aminoindanyl)pyrimidine (30). 2-Hydroxychroman[24] (0.24gm, 1.61 mmole) was combined with **29** (0.46gm, 1.47 mmole) in a mixture of ethanol (10 mL) and glacial acetic acid (0.5 mL) at room temperature. To the resulting suspension was added $NaCNBH_3$ (0.23gm, 3.53 mmol) in portions over 9 min. After 50 min the reaction mixture was partitioned between brine and ethyl acetate. The aqueous phase was extracted with ethyl acetate and the combined organics were dried with $MgSO_4$ and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (ethyl acetate−hexane−methanol): 0.39 g, 59% yield; ^{1}H NMR (DMSO-d_6) δ 8.82 (1H, bs), 7.68 (2H, d, J = 8.5 Hz), 7.19−7.2 (2H, cm), 7.11−7.14 (2H, cm), 7.05 (1H, d, J = 7.3 Hz), 6.96−6.99 (1H, t), 6.77 (2H, d, J = 8.5 Hz), 6.69−6.72 (1H, t), 6.58 (1H, bs), 6.55 (2H, d, J = 8.5 Hz), 6.13 (1H, s), 5.56 (1H, bs), 5.42 (2H, bs), 4.65−4.69 (1H, cm), 3.30−3.25 (2H, dd, J = 7.3, 15.9 Hz), 3.06−3.11 (2H, cm), 2.82−2.86 (2H, dd, J = 6.1, 15.9 Hz), 2.62−2.65 (2H, t), 1.81−1.87 (2H, cm); MS (M + H) 452.

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Supporting Information Available: Data for compound purity and structural identity. This information is available free of charge via the Internet at http://pubs.acs.org.

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