Femtomolar Transition State analogue Inhibitors of 5′-Methylthioadenosine/S-Adenosylhomocysteine Nucleosidase from *Escherichia coli*

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*Escherichia coli* 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) hydrolyzes its substrates to form adenosine and 5-methylthioribose (MTR) or S-ribosylhomocysteine (SRH). 5′-Methylthioadenosine (MTA) is a by-product of polyamine synthesis and SRH is a precursor to the biosynthesis of one or more quorum sensing autoinducer molecules. MTAN is therefore involved in quorum sensing, recycling MTA from the polyamine pathway via adenosine phosphoribosyltransferase and recycling MTR to methionine. Hydrolysis of MTA by *E. coli* MTAN involves a highly dissociative transition state with ribooxacarbenium ion character. Inmonilbitol mimics of MTA at the transition state of MTAN were synthesized and tested as inhibitors. 5′-Methylthio-Immucillin-A (MT-ImmA) is a slow-onset tight-binding inhibitor giving a dissociation constant of 2 pM. DADMe-ImmA binds with a Ki value of 2 pM. Replacing the 5′-methyl group with other hydrophobic groups gave 17 transition state analogue inhibitors with dissociation constants from 10−12 to 10−14 M. The most powerful inhibitor was 5′-p-CI-phe-thio-DADMe-Immucillin-A (pCIpHT-DADMe-ImmA) with a Ki value of 47 fm (47 × 10−15 M). These are among the most powerful non-covalent inhibitors reported for any enzyme, binding 9–91 million times tighter than the MTA and SAH substrates, respectively. The inhibitory potential of these transition state analogue inhibitors supports a transition state structure closely resembling a fully dissociated ribooxacarbenium ion. Powerful inhibitors of MTAN are candidates to disrupt key bacterial pathways including methylation, polyamine synthesis, methionine salvage, and quorum sensing. The accompanying article reports crystal structures of MTAN with these analogues.

5′-Methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) functions at two steps in bacterial pathways related to polyamine biosynthesis, quorum sensing, methylation, and purine and methionine salvage reactions (Fig. 1; see Refs. 1–4). It catalyzes the physiologically irreversible hydrolysis of 5′-methylthioadenosine (MTA) to adenosine and 5-methylthio-o-ribose (MTR). The product adenosine is subsequently recycled into the adenosine nucleotide pool by the widely distributed adenosine phosphoribosyltransferase (5), and the 5-methylthio-o-ribose is subsequently phosphorylated to 5-methylthio-o-ribose 1-phosphate and converted into methionine (6). MTA is a by-product of the reactions involving sequential transfers of the aminopropyl group from decarboxylated S-adenosylmethionine to form spermidine and spermine (although spermine is absent in *Escherichia coli*). Polyamine synthesis is sensitive to product inhibition by MTA, with inhibition constants reported to be 0.3 μM for bovine spermine synthase (7) and 50 μM for rat spermine synthase (8). Inhibition of MTAN is therefore expected to inhibit polyamine biosynthesis and the salvage pathways for adenosine and methionine. Another function of MTAN in bacteria is generation of S-ribosylhomocysteine (SRH) from SAH. SRH is a precursor to the biosynthesis of one or more quorum sensing autoinducer molecules involved in expression of the enzymes for biofilm formation, exotoxin synthesis, and antibiotic resistance factors (9–14). Previously characterized nucleoside and nucleotide N-ribosyl hydrolases proceed through transition state structures in which the N-ribosidic

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1 The abbreviations used are: MTAN, 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase; MTA, 5′-methylthioadenosine; SAH, S-adenosylhomocysteine; MTR, methylthioribose; MT-ImmA, MT-Immucillin-A, (1S)-1-[9-deazaadenin-9-yl]-1,4-dideoxy-1,4-imino-5-methylthio-o-ribitol; pCIpHT-ImmA, (1S)-[5-[4-chlorophenylthio]-1-[9-deazaadenin-9-yl]-1,4-dideoxy-1,4-imino-o-ribitol; pCIPHT-DADMe-ImmA, (3R,4S)-4-(4-chlorophenylthio)-1-[9-deazaadenin-9-yl]-1,4-dideoxy-1,4-imino-5-(3-methylthiohydroxypyridinyl)-o-ribitol; MT-DADMe-ImmA, 5′-methylthio-DADMe-Immucillin-A, (3R,4S)-1-[9-deazaadenin-9-ylmethyl]-3-hydroxy-4-(methylthiomethyl)pyrrolidine; BnT-DADMe-ImmA, (3R,4S)-4-(benzylthiomethyl)-1-[9-deazaadenin-9-ylmethyl]-3-hydroxy-4-(methylthiomethyl)pyrrolidine; pCIPHT-DADMe-ImmA, (3R,4S)-4-(4-chlorophenylthio)-1-[9-deazaadenin-9-yl]-1,4-dideoxy-1,4-imino-5-(3-methylthiohydroxypyridinyl)-o-ribitol; MT-DADMe-8-azaImmA, (3R,4S)-1-[8-aza-9-deazaadenin-9-ylmethyl]-4-(benzylthiomethyl)-3-hydroxy-pyrrolidine; Boc-t-butoxycarbonyl. The IUPAC names of other inhibitors are readily derived from the examples given above. Ki, dissociation constant for the equilibrium complex of enzyme-inhibitor following slow-onset inhibition.
bond is partially cleaved at the transition state without significant bond order to the attacking water nucleophile (15–17). The Immucillin transition state analogues designed for these enzymes were modified to incorporate chemical features of MTA and were the starting point for our inhibitor design program. Kinetic isotope effect analysis for MTAN has established the transition state structure to be more closely related to a highly dissociated ribooxacarbenium ion.² Highly dissociated transition states for N-ribosyltransferases are closely related to the human and malarial purine nucleoside phosphorylases and to ricin, which have all been shown to form highly dissociated S₃N₁-like transition states (18–20). The second-generation DADMe-Immucillins are closer mimics of highly dissociated N-ribosyltransferase transition states (20, 21), and we modified the DADMe-Immucillins to incorporate features of MTA for the second-generation MTAN inhibitors (Fig. 2). Transition state structures derived from kinetic isotope effect analysis have proven to provide robust atomic blueprints for the design of transition state analogues (15, 21–23). In contrast, protein structural similarity has not been a strong predictor of transition state structure. For example, the catalytic sites of human and bovine purine nucleoside phosphorylases are identical within the error of x-ray diffraction experiments, yet the transition states differ considerably (20). Thymidine phosphorylase, another ribosyltransferase, was predicted from structural and computational analysis to have a dissociative transition state, but kinetic isotope effect analysis established a near-classical S₃N₂ transition state (24, 25). Recent analyses have implicated dynamic motion as an important component of transition state formation. The only dependable way to understand enzymatic transition states is with methods such as kinetic isotope effect analysis that are independent of dynamics (21).

Transition state analogue inhibitors are designed from the hypothesis that chemically stable analogues that mimic geometric and molecular electrostatic features of the transition state will be bound to the enzyme tighter than the substrate by the factor equal to the catalytic rate of acceleration imposed by the enzyme (22, 23). Transition state analogues with nearly irreversible binding can hypothetically be designed for enzymes with typical catalytic rate enhancements (10¹⁰ to 10¹⁵). Nucleoside hydrolases enhance reaction rates by 10⁻¹². With Kᵣ values of 0.43 and 4.3 μM for MTA and SAH, respectively (26–28), perfectly designed transition state analogues for MTAN should have dissociation constants of 10⁻¹⁵ to 10⁻¹⁸ μM. However, it is impossible to design perfect transition state analogues, since the actual enzymatic transition state has nonequilibrium bond lengths and charges that cannot be accurately copied in chemically stable molecules.

²V. Singh, S. Nunez, J. E. Lee, P. L. Howell, and V. L. Schramm, unpublished results.

FIG. 1. Pathways connecting polyamine synthesis, adenine salvage, methionine salvage, and tetrahydrofuran synthesis in bacteria. The tetrahydrofurans are precursors for the synthesis of autoinducer-2 molecules (AI-2), while N-acyl-substituted homoserine lactones are autoinducer-1 molecules (AI-1). Both are signaling molecules for quorum sensing. MTAN is encoded by the pfs gene in E. coli and catalyzes two reactions in this cycle. The diagram is adapted from the summary provided in Ref. 9. SAM, S-adenosylmethionine; RH, ribosyl-homocysteine; THF, tetrahydrofolate.

FIG. 2. MTAN-catalyzed hydrolysis of MTA and the dissociative transition state of the reaction (A). Features of the transition state that distinguish it from substrate are shown in blue. 5'-Thio substituted Immucillin-A molecules mimic an early transition state where the bond between the ribosyl and adenine groups retain partial bond order. Features important for transition state recognition are shown in blue (B). 5'-Thio-substituted DADMe-Immucillin-A molecules mimic a late transition state where the ribosyl cation is fully dissociated from the adenine leaving group, a distance of ~3 Å (B). In fully dissociated ribosyl transition states, the site of carboxonium formation is at C1', and the N1' mimics this geometry. The methylene bridge positions the leaving group at an appropriate distance from the ribooxacarbenium ion site; 9-deazaadenine provides a carbon-carbon bridge for chemical stability and to increase the pKᵣ at N7. Features important for transition state recognition are shown in blue.
We synthesized putative transition state analogues for MTAN that mimic properties of both partially and fully dissociated S_{2,1} transition state structures. Efficient transition state analogue inhibitors include ribooxacarbene ion (carbocation) mimics and a 9-deazaadenine group to mimic the elevated pKa of the ribooxacarbene ion. A stable C–C riboside bond replaces the N-ribosidic bond of substrates and an elevated pK_{a} at the N7 position is accomplished by the use of 9-deazaadenine. Iminoribitol mimics resemble early transition states where significant N-ribosidic bond order remains at the transition state. To mimic a fully dissociated ribooxacarbene transition state, the DADMe-Immmiculin family was synthesized. These analogues include the pyrrolidine moiety as the ribooxacarbene ion mimic and a methylene bridge between the ribooxacarbene ion mimic and the 9-deazaadenine to provide a geometric approximation of the N-ribosidic bond distance for a fully dissociated transition state. Chemical stability of the pyrrolidine nitrogen is achieved by three neighboring methylene groups, generating analogues resembling 2'-deoxyribonucleotides. This construct expressed a protein with six histidines at the N-terminus and was purified by Ni-NTA chromatography (28, 31). The histidine tag was removed by chemotryptic digestion following purification. The digested protein has a linker region of ten amino acid residues at its N-terminus. The purified protein was analyzed by purity by SDS-PAGE gels stained with Coomassie Blue and was stored at 15 °C following flash freezing in dry ice-acetone.

**Materials and Methods**

**Enzyme Preparation**—The fragment containing full-length *E. coli* MTAN was obtained by EcoRI/NcoI digestion of pXMx (28) and cloned into a pPROEX HTa expression vector. The vector was transformed into *E. coli* strain TOP10F'. The transformed cells were cultured in LB medium containing 100 μg/ml ampicillin at 37 °C, and MTAN was expressed by induction with 1 mM isopropyl β-D-thiogalactopyranoside. The expressed protein was purified by a nickel affinity chromatography column (28, 31). The resultant product with ammonia, and global deprotection to afford the desired product. Structure and stereochemistry of pT-DADMe-Immmiculin (23) was confirmed by mass spectrometry and NMR.

Femtomolar Inhibitors of MTAN

Femtomolar inhibitors with femtomolar dissociation constants are rare in enzymology, making these compounds among the most powerful noncovalent enzyme inhibitors yet reported.

**Synthetic Chemistry of Immucillin and DADMe-Immmiculin, Inhibitor Synthesis**—The inhibitors pCIPt-Immmiculin A (1), pTDT-Immmiculin A (2), mTDT-Immmiculin A (3), pCIPt-Immmiculin A (4), mCIPt-Immmiculin A (5), pFIPt-Immmiculin A (6), ET-FIPt-Immmiculin A (8), PfIPt-Immmiculin A (9), PhIPt-Immmiculin A (10), 5'-d-ET-Immmiculin A (11), MT-Immmiculin A (12), OH-ET-Immmiculin A (13), NIPt-Immmiculin A (14), 5'-Me-Immmiculin A (15), and 5'-d-Immmiculin A (16) were prepared as described previously (30).

DADMe-Immmiculin (32) and pIPt-DADMe-Immmiculin (36) were synthesized via selective methanesulfonfylolation of the hydroxymethyl group of the previously reported (3R,4R)-N-tert-butoxy carbonyl-3-hydroxy-4-hydroxy methylpyrrolidine hydrochloride (3R,4S)-3-hydroxy-4-(methylthiomethyl)pyrrolidine hydrochloride, (3R,4S)-3-hydroxy-4-(3-chlorophenylthiomethyl)pyrrolidine hydrochloride, (3R,4S)-3-hydroxy-4-(4-methylthiomethyl)pyrrolidine hydrochloride, (3R,4S)-3-hydroxy-4-(4-ethylthiomethyl)pyrrolidine hydrochloride, (3R,4S)-3-hydroxy-4-(propylthiomethyl)pyrrolidine hydrochloride, (3R,4S)-3-hydroxy-4-(3-cyclohexane-1-thiomethyl)pyrrolidine hydrochloride, (3R,4S)-3-hydroxy-4-(1-propyl-1-thiomethyl)pyrrolidine hydrochloride, or (3R,4S)-3-hydroxy-4-(prop-2-ylthiomethyl)pyrrolidine hydrochloride respectively. Conversion of these amine hydrochlorides to the DADMe-Immmiculin-A derivatives was achieved via a Mannich reaction with formaldehyde and 9-deazaadenine described previously.

BuN-Pz-DADMe-Immmiculin A (23) was synthesized via the reductive amination of the previously reported aldehyde 8-aza-9-deaza-9-formyl-6-O-methyl-8-tetrahydropropylyoxantheine (34) and the aforementioned (3R,4S)-3-hydroxy-4-(benzylthiomethyl)pyrrolidine hydrochloride with sodium cyanoborohydride, displacement of the 6-methoxy group of the resultant product with ammonia, and global deprotection to afford the desired product. Structure and stereochemistry of pT-Pz-DADMe-Immmiculin (23) was confirmed by mass spectrometry and NMR.

Treatment of (3R,4S)-3-Hydroxy-4-(methoxymethyl)pyrrolidine hydrochloride and (3R,4R)-3-Hydroxy-4-(benzoxymethyl)pyrrolidine hydrochloride were prepared via selective protection of the C5 hydroxyl of (3R,4R)-N-tert-butoxy carbonyl-3-hydroxy-4-hydroxy methylpyrrolidine as a tert-butyldimethylsilyl ether, protection of the remaining hydroxyl with methoxyethyl acetate, removal of the silyl protecting group, alkylation of the C5 hydroxyl, followed by formation of the tert-butoxy carbonyl protecting group with methanolic HCl to afford the hydrochloride salt of the amine. Conversion of these amine hydrochlorides to (3R,4R)-1-[9-deazaadenin-9-yl)methyl]-3-hydroxy-4-(methoxymethyl)pyrrolidine and (3R,4R)-4-(benzoxymethyl)-1-[9-deaza-adenin-9-yl)methyl]-3-hydroxy-4-hydroxy methylpyrrolidine, respectively, was achieved by a Mannich-type reaction with formaldehyde and 9-deazaadenine, via a previously reported general method for the conversion of the C5 hydroxyl of (3R,4R)-N-tert-butoxy carbonyl-3-hydroxy-4-hydroxy methylpyrrolidine with sodium periodate afforded an intermediate aldehyde which was treated, without further purification, with the appropriate Wittig salt, followed by hydrogenation of the EZ mixture of stereoisomers and removal of the tert-butoxy carbonyl protecting group with methanolic HCl to afford the hydrochloride salt of the aforementioned amines.

Homocysteine in the presence of methionine, followed by removal of the thiolactone protecting group with methanesulfonfylolation of the hydroxymethyl group with sodium periodate and global deprotection of the N-Boc protected thiolactone of homocysteine in the presence of methionine, followed by removal of the trichloroethoxycarbonyl (Troc) protecting group and conversion of the 3-(3R,4S)-3-hydroxy-4-(2-phenylethyl)pyrrolidine was achieved as described above.

Homocysteine-DADMe-Immmiculin A (34) was synthesized as a selective methanesulfonfylolation of the hydroxymethyl group of the previously reported (3R,4R)-N-tert-butoxy carbonyl-3-hydroxy-4-hydroxy methylpyrrolidine (34), removal of the Boc protecting group with 4 M HCl in dioxane, followed by reprotection of the amine with 2,2,2-trichloroethanol chloride formate. Displacement of the methanesulfonfyl group with the N-Boc protected thiolactone of homocysteine in the presence of methionine, followed by removal of the trichloroethoxycarbonyl (Troc) protecting group and conversion of the 3-(3R,4S)-3-hydroxy-4-(2-phenylethyl)pyrrolidine hydrochloride which underwent a Mannich-type reaction with formaldehyde and 9-deazaadenine, via a previously reported general method to afford, after global deprotection of the Mannich base, Homocysteine-DADMe-Immmiculin A (34) (35).
group of (3R,4S)-N-tert-butoxycarbonyl-3-hydroxy-4-hydroxymethylpyrrolidine (34) followed by displacement of the sulfonate group with thioacetate to afford (3R,4S)-N-tert-butoxycarbonyl-3-hydroxy-4-ace-
ylation of pyrrolidine. This compound was deacetylated with sodium methoxide in the presence of either cyclohexylmethyl methan-
sulfonate or cyclohexyl, cyclopentyl, or cyclobutyl bromide; and the corresponding (3R,4S)-3-hydroxy-4-(alkylthiomethyl)pyrrolidine hydro-
chlorides were isolated after removal of the Boc group with methanolic HCl. Conversion of these amine hydrochlorides to the DADMe-Immu-
cillin-A derivatives was achieved via a Mannich reaction with formal-
dehyde and 9-deazadenine as described previously (35).

pCIPHT-DADMe-Indole (48) was prepared via the reductive amination of (3R,4S)-3-hydroxy-4-(4-chlorophenylthiomethyl)pyrrolidine hy-
drochloride and the commercially available indole-3-carboxaldehyde with sodium cyanoborohydride.

MT-DADMe-3-denza-ImmA (49) was synthesized via a Mannich re-
action with formaldehyde, 3-hydroxy-4-thiomethylpyrrolidine and N-Ac-3,9-dideazaadenine, followed by deprotection of the acyl group. The N-Ac-3,9-dideazaadenine moiety was prepared from 2-hydroxy-4-
methyl-3-nitropyridine. Treatment of this hydroxy.pyrididine with POCI3 provided the chloride, which was displaced with ammonia to afford 2-amino-4-methyl-3-nitropyridine. The amino group was acylated be-
fore reaction with Brederick’s reagent, which provided the enamine.

This enamine was then reductively cyclized with zinc dust in acetic acid to give N-Ac-3,9-dideazaadenine. Me-Sulf oxide-ImmA (44), as a ~1:1 mixture of diastereomers, was prepared by treating MT-ImmA (12) with 30% hydrogen peroxide. In a similar fashion, hydrogen peroxyde treatment of MT-DADMe-ImmA (32) for a short time gave Me-Sulf oxide-
DADMe-ImmA (46) as a single diastereomer but of undetermined ste reoisomer at sulfur. Hydrogen peroxide treatment of MT-
DADMe-ImmA (32) for a longer time period gave a separable mixture of Me-Sulfone-DADMe-ImmA (47) and Me-Sulf oxide-DADMe-ImmA (45) (as an ~1:1 mixture of diastereomers).

**Determination of Inhibition Constants**—Direct spectrophotometric assay involved monitoring the conversion of MTA to adenine by E. coli MTAN at 275 nm. A coupled assay with xanthine oxidase monitored the formation of 2,8-dihydroxyadenine produced by oxidation of adenine by xanthine oxidase at 293 nm. Under the assay conditions, the $K_m$ for conversion of MTA to adenine is 1.6 mM$^{-1}$, and it is 15.2 mM$^{-1}$ for the conversion of MTA to 2,8-dihydroxyadenine at pH 7.0 in 0.1 M HEPES buffer. Concentrations of most inhibitors were determined by ultraviolet absorption using the extinction coefficient of 9-deazadenine of 8.5 mM$^{-1}$ cm$^{-1}$ at 275 nm at pH 7.0. Solutions of pCIPHT-DADMe-Indole (48), MT-DADMe-3-denza-ImmA (49), BnT-Pz-DADMe-ImmA (23), MT-Pz-ImmA (7), and formycin A (20) were made from sample weight. Concentration of MT-ImmA (19) was determined with the extinction coefficient of 9-deazahypoxanthine of 9.54 mM$^{-1}$ cm$^{-1}$ at 261 nm (36).

The reactions for measuring the inhibition constants for Immucillins were initiated by adding the enzyme (1–5 nM) to the reaction mixture, typically containing 200 μM MTA, 100 mM HEPES, pH 7.5, 50 mM KCl and varying amount of inhibitor concentration in 1-ml reaction volume at 25 °C. For tight binding DADMe-Immucillins having affinities in the low picomolar to femtomolar range, MTA concentrations of 2–3 mM were required to observe appropriate reaction rates. The coupled assay with xanthine oxidase was used with high concentrations of substrate. Reaction mix-
tures for the xanthine oxidase-coupled assay contained 100 mM HEPES, 50 mM KCl, 2–3 mM MTA, 0.5 unit/ml xanthine oxidase, and varying amount of inhibitor concentrations in a reaction volume of 1 ml at 25 °C. The reactions were initiated by adding 1–5 nM MTAN. Controls having no inhibitor and no enzyme were included in all experiments. The $K_i$ values for inhibitors were obtained by fitting the initial rate and inhibitor concen-
tration to the following expression of competitive inhibition

$$V_o = \frac{K_o + [S]}{K_o + [S] + K_i[I]/K_i}$$ (Eq. 1)

where $V_o$ is the initial rate in the presence of inhibitor, and $V_{o0}$ is the initial rate in the absence of inhibitor, $[I]$ is the inhibitor concentration, and $[S]$ is the substrate concentration. This expression is valid only under the condition where the inhibitor concentration is 10 times greater than the enzyme concentration. However, when the inhibitor concentration was only a few fold greater than the enzyme concentration, the effective inhibitor concentration was obtained by the expression,

$$I' = 1 - \left(1 - V_o/V_{o0}\right) E_i$$ (Eq. 2)

where $I'$ is the effective inhibitor concentration, $V_{o0}$ and $V_o$ are the initial rate in the presence and absence of inhibitor, and $E_i$ is the total enzyme concentration. Most tight binding transition state analogue inhibitors displayed a second linear reaction rate following slow-onset tight binding of the complex, indicating that they had reached thermodynamic equilib-
rium with the enzyme. Examples are given in the slow-onset inhibition curves of Figs. 3–5. The equilibrium dissociation constant ($K_i^*$) was obtained by fitting the rates to the following equation of competitive inhibition,

$$V_o = \frac{K_o + [S]}{K_o + [S] + K_o[I]/K_i^*}$$ (Eq. 3)
Comparison of inhibition constants throughout this work are values representing true thermodynamic equilibrium (dissociation constants) between enzyme and inhibitor. In cases where both $K_i$ and $K_i^*$ are reported, $K_i^*$ represents this dissociation constant. In cases where only $K_i$ is reported, $K_i$ is the dissociation constant. Thus, it is correct to compare $K_i$ and $K_i^*$ values in some cases.

**RESULTS AND DISCUSSION**

**MTAN Transition State Analogue Inhibitor Design**—E. coli MTAN hydrolyzes the N-ribosidic bond of its substrates with highly developed ribooxacarbenium-ion character at the transition state. Other N-ribosyl transferases including purine nucleoside phosphorylases from human and malarial sources, ricin A-chain and human MTA phosphorylase are also characterized by ribooxacarbenium ion character at their transition states (18, 20, 29). Transition states for purine N-ribosyltrans-

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**Fig. 4. Inhibition of E. coli MTAN by pChPhT-ImmA [1].** An example of data analysis for a slow-onset, tight-binding Immucillin derivative. The rate traces in the upper panel are from the direct assay that monitored the difference spectra between MTA and adenine. Initial rates of inhibition were used to evaluate $K_i$ from fits to the equation for competitive inhibition as indicated in the methods (middle panel). Following slow-onset inhibition, a second set of slopes is observed (80–100 min), and these are fitted to the equation for competitive inhibition to evaluate $K_i^*$, the equilibrium dissociation constant (lower panel). Details of the analysis and equations are given in the methods. Similar methods were used to measure other inhibitors shown in Figs. 6 and 7.

**Fig. 5. Inhibition of E. coli MTAN by cHeptylT-DADMe-ImmA [41].** An example of data analysis for a slow-onset, tight binding 5'-thio-substituted DADMe-Immucillin derivative. The reaction rate and slow-onset inhibition were monitored by the conversion of MTA to 2,8-dihydroxyadenine in a coupled reaction with xanthine oxidase (upper panel). The coupled assay permits use of high substrate concentration (2.0 mM, 4,650 $\mu$M $K_m$ for MTA) to compete against these powerful inhibitors. Values of $K_i$ (middle panel) and $K_i^*$ (lower panel) were obtained from the initial (0–5 min) and the final rates (25–30 min). Similar experiments were used to measure inhibition by slow-onset tight binding inhibitors shown in Fig. 8.

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3 Slow-onset, tight binding inhibition is common with transition state analogues (43). In the first phase, inhibitor binds reversibly to form the $EI$ complex, defined by the dissociation constant $K_i$. In the second step, $EI$ undergoes conformational tightening ($EI \rightarrow EI^*$) where the inhibitor is bound more tightly. The equilibrium between free enzyme and the $EI^*$ complex is defined by $K_i^*$, the equilibrium dissociation constant.
favorable H-bond (see Refs. 38 and 39 and the accompanying article (40)).

MT-ImmA [12] was designed to mimic an early transition state with MTA as substrate. It is a powerful inhibitor exhibiting slow-onset tight-binding inhibition with a $K_i$ value of 130 pM and the $K_i^*$ equilibrium constant of 77 pM (Fig. 6). The $K_{in}$ value for MTA is 0.43 µM to give a $K_{in}/K_i^*$ value of 5,600 for MT-ImmA [12]. The increase in apparent affinity of MT-ImmA [12] can be partially attributed to a H-bond between the N7 and Asp197. An additional H-bond between the N8 in MT-Pz-ImmA [7] and Ser76 Oγ (38) improves its $K_{in}/K_i^*$ value to 16,500.

The pocket in MTAN that binds the 5'-methylthio group is hydrophobic and this part of the catalytic site is composed of flexible amino acid side chains. Substrate specificity demonstrates that the region has sufficient conformational flexibility to accept both methylthio and homocysteine groups, although the $K_{in}$ for SAH [39] is 10-fold greater than that for MTA [17] (4.3 versus 0.43 µM). The 5'-methylthio site can accommodate both polar and non-polar groups as indicated by the binding of SAH [39] and 5'-substituted substrates such as 5'-(p-nitrophenoxythio)adenosine (26). Incorporating other hydrophobic and halogenated aliphatic and aromatic groups at the 5'-thio position of 5'-thio-ImmA increased the binding affinity to give dissociation constants of 6 to 12 pM in pCIPHT-ImmA [1], pToIT-ImmA [2], mToIT-ImmA [3], and BnT-ImmA [4]. Aromatic and small nonpolar, aliphatic residues that surround the 5'-methylthio binding site include Met9, Ile36, Val105, Phe205, Tyr107, Pro113, and Phe197, and these are proposed to interact with the 5'-hydrophobic substituents. Among the 5'-substituted Immucillin-A family, pCIPHT-ImmA [1] with $K_i^*$ of 2 pM is the tightest binding inhibitor with $K_{in}/K_i^*$ of 214,800 with respect to MTA [17] and 2,148,000 relative to SAH [39]. The slow-onset inhibition pattern is readily observed in the inhibition pattern (Fig. 4).

Design of bacteria-specific inhibitors requires discrimination against human enzymes catalyzing similar reactions. In humans there is no MTAN and 5'-methylthioadenosine phosphorylase (MTAP) is the only enzyme capable of metabolizing MTAN. Comparisons between MTAN and MTAP reveal that MTAP has a more restricted 5'-alkythio binding site, thus accounting for the weaker binding of BnT-ImmA [4] to MTAP than to MTAN with dissociation constants of 26,000 and 12 pM, respectively (29, 30, 41). This provides a specificity factor of 2,200 for the bacterial enzyme and provides potential for targeting bacterial MTAN without inhibiting human MTAP.

**DADMe-Immucillin Inhibitor Design for MTAN**—The DADMe-Immucillins (Fig. 7) were originally designed to match the geometry and molecular electrostatic features of fully dissociated riboxygenium ion transition states such as those found in human purine nucleoside phosphorylase (20). MTAN from E. coli also has a fully dissociated transition state and it would be anticipated that the DADMe-Immucillins would bind better than the Immucillins. MT-DADMe-ImmA [32] has $K_i$ and $K_i^*$ values of 48 and 2 pM, respectively, with a $K_{in}/K_i^*$ of 215,000 with respect to MTA and 2,150,000 with respect to SAH. The 5'-methylthio binding pocket readily accommodates larger alkyl groups including ethyl, propyl, and butyl. The affinity increases with the increase in length of the 5'-alkyl chain. BnT-DADMe-ImmA [29], PrT-DADMe-ImmA [26], and BuT-DADMe-ImmA [22] bind with $K_i^*$ values of 950, 580, and 296 fm, respectively. BuT-DADMe-ImmA [22] is the second most powerful inhibitor of the DADMe-Immucillins. It

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*Immuocillin-H is (1S)-1-(9-deazahypoxanthin-9-yl)-1,4-dideoxy-1,4-imino-2-ribitol and has been shown to have a $pK_a > 10$ at pH 7 (37). MT-ImmA is chemically similar in the 9-deazaadenine ring and is expected to have similar $pK_a$. The $K_{in}$ values for MTAN are not dissociation constants, since $K_{in}$ contains rate terms as well as terms for the dissociation constant for substrate. Because of this relationship, $K_{in}/K_i^*$ only approximates the ratio of binding affinity relative to substrate.*
DADMe-ImmA shows slow-onset inhibition with Immucillins as was also seen for the Immucillins. Incorporating polar groups, like chloro and para, to the thiol substituted DADMe-Immucillins are compared with the pKa25 binds 14,500,000-fold more tightly than SAH [41]. The accompanying article (40) describes the structure of E. coli MTAN with MT-DADMe-ImmA [32] and demonstrates that the N1' cation of MT-DADMe-ImmA [32] provides a favorable electrostatic/H-bond interaction between N1' and the nucleophilic water at a distance of 2.7 Å.

Comparison of Immucillins and DADMe-Immucillins with the same 5'-thio substituents indicates the thermodynamic benefit of increasing the distance between 9-deazaadenine and the riboxacarbenium mimics. pClPhT-ImmA [1] and pClPhT-DADMe-ImmA [21] gave dissociation constants of 2 pm and 47 fm, a factor of 43 tighter binding for the DADMe inhibitor. Similar values are seen in comparing pFPnT-ImmA [6] and pFPnT-DADMe-ImmA [25] where the DADMe derivative binds 36-fold more tightly. Likewise, PhT-DADMe-ImmA [30] binds 16-fold tighter than PhT-ImmA [10] (Figs. 6 and 7). Finally, the MT, mClPhT, and EtT groups achieve an extra 26-, 27-, and 28-fold affinity, respectively, from being in the DADMe context. BnT-ImmA [4] and BnT-DADMe-ImmA [24] have Ki* values of 12 pm and 460 fm, respectively, a factor of 26. The consistent affinity ratio in comparing Immucillins and DADMe-Immucillins indicates that the methylene bridge and DADMe feature improves binding by ~2 kcal/mol.

Changes in the purine base can also be interrogated for the effects on binding affinity. Thus, BnT-Pz-DADMe-ImmA [23] binding is equivalent to that for BnT-DADMe-ImmA [24]. The 3-fold improved binding of MT-Pz-ImmA [7] relative to MT-ImmA [12] is consistent with a favorable hydrogen bond between N8 and Ser10 at the active site or may reflect a more favorable H-bond at N7 due to its pKα (9.6 for N7 of [7]). Incorporating polar groups, like chloro and para, to the thiol significantly improves the binding affinity of DADMe-Immucillins as was also seen for the Immucillins. pClPhT-DADMe-ImmA [21] shows slow-onset inhibition with Ki and Ki* values of 2.6 pm and 47 fm and binds 40 times more tightly than PhT-DADMe-ImmA [30] (Fig. 7). It is the tightest binding inhibitor of the DADMe-Immucllins against E. coli MTAN with Ks/Ki* values of 9.1 and 91 million relative to MTA [17] and SAH [39], respectively. Binding affinity 91 million times tighter than substrates is unprecedented for noncovalent enzymatic inhibitors. This corresponds to over 12 kcal/mol favorable binding energy relative to SAH. The improved binding of DADMe-Immucillins indicates that they are closer mimics of the transition state by 2 kcal/mol than are the Immucillins. The N1' pyrrolidine nitrogen in DADMe-Immucillins has a pKα near 9.0 (42), making this group more cationic than the N4'-imino group in Immucillins (pKα of 6.9). The accompanying article (40) describes the structure of E. coli MTAN with MT-DADMe-ImmA [32] and demonstrates that the N1' cation of MT-DADMe-ImmA [32] provides a favorable electrostatic/H-bond interaction between N1' and the nucleophilic water at a distance of 2.7 Å.

**Cycloalkyl Substitutions at the 5'-Thiol of DADMe-ImmA**—The residues in the active site of E. coli MTAN that interact with the 5'-thiol group of substrate analogues include Met9, Ile91, Val105, Phe103, Tyr107, Pro113, and Phe207 (41). These residues are predominantly hydrophobic and interact favorably with 5'-hydrophobic substituents of DADMe-Immucillins. Thus, MTAN shows a 2.5 times higher binding affinity toward cyclohexyl groups (cHexT-DADMe-ImmA [27]) relative to the less hydrophobic benzyl groups (PhT-DADMe-ImmA [30]). DADMe-Immucillins of varying 5'-hydrophobicity were synthesized by increasing or decreasing the size of the cycloalkyl group (Fig. 8). MTAN binds less tightly to inhibitors with small 5'-groups like cButylT-DADMe-ImmA [43] and cPentyT-DADMe-ImmA [42], with a Ki* of 2.2 and 1.5 pm, respectively, than to cHexT-DADMe-ImmA [27], which binds MTAN with the Ki* of 740 fm. cHeptylT-DADMe-ImmA [41] binds less tightly with a Ki* of 1.3 pm (Fig. 8). Increasing the distance between the 5'-thiol and a cyclohexyl group by incorporating a methyl group had no effect, thus the binding affinity of cHexMeT-DADMe-ImmA [40] (Ki* of 670 fm) is not significantly different from cHexT-DADMe-ImmA [27] (740 fm; Fig. 8). Tight binding in the aromatic substituents depends on the placement of the hydrophobic group. Although the effects are not large, incorporation of a methylene bridge to convert PhT-ImmA [10] to BnT-ImmA [4] or PhT-DADMe-ImmA [30] to BnT-DADMe-ImmA [24] creates stronger binding in both cases, presumably by better interactions with the hydrophobic residues. Cyclic alkyl groups eliminate favorable p-π base stacking or herringbone aromatic-aromatic interactions with Phe105, Tyr107, and Phe207. Because of the small differences in binding energy between the 5'-cyclic alkyl and aryl substituents we con-
clude that aromatic-aromatic interactions are secondary to the general hydrophobic effect.

Substitutes of Immucillins and DADMe-Immucillins—Residues close to the thiomethyl in the active site of E. coli MTAN are hydrophobic in nature. Incorporating sulfoxides and sulfones at the 5'-carbon of DADMe-Immucillins decreased their binding affinity, establishing that these polar groups are energetically unfavorable. Me-Sulfoxide-DADMe-ImmA [46] and Me-Sulfone-DADMe-ImmA [47] bind 500–1,000-fold less tightly than MT-DADMe-ImmA [32] (Fig. 9). A 1:1 diastereomeric mixture of Me-Sulfoxide-DADMe-ImmA [45] gave a $K_i$ of 1.0 nM, whereas a purified diastereomer of unknown orientation binds with the $K_i$ of 1.5 nM. These $K_i$ values suggest a preference for the other diastereomer. Me-Sulfone-DADMe-ImmA [47], which has two oxygens near the hydrophobic pocket, binds even less tightly with a $K_i$ of 2.0 nM, a 1,000-fold decrease in binding affinity compared with MT-DADMe-ImmA [32].

Substitution of the Thioether in DADMe-Immucillins with Oxygen or Carbon—BnT-DADMe-ImmA [22] gave a $K_i^*$ of 460 pm, one of the more powerful inhibitors. Substituting sulfur with oxygen as in BnO-DADMe-ImmA [35] decreases the binding affinity by ~20-fold to bind with a $K_i^*$ of 9.0 pm. 5’-dBN-DADMe-ImmA [38] is an analogue of PhT-DADMe-ImmA [30] and has a methylene group replacing the sulfur. This replacement alters the $K_i^*$ values from 2 pm to 1 nM, a 500-fold decrease in affinity (Fig. 7). Similar trends in the binding constants were also observed for MT-DADMe-ImmA [32] ($K_i^* = 2.0$ pm), 5’-dEt-DADMe-ImmA [33] ($K_i^* = 6$ pm), and MeO-DADMe-ImmA [37] ($K_i^* = 62$ pm). Likewise, MT-ImmA [12] binds more favorably than 5’-Me-ImmA [15] by a factor of 130. The electron-rich oxygen is responsible for this difference, since replacing the thioether with a methylene bridge (5’-dEt-ImmA [11]) is approximately equivalent in binding to MT-ImmA [12].

Substitutions in the 9-Deazaadenine Ring of the Immucillins—The adenine binding pocket of E. coli MTAN is composed of Phe151, Ile152, Ser196, Asp197, and Ala199 to form an extensive hydrogen bonding network with each other and with N1, N7, and the exocyclic amino N6 group (38). Disruption of this H-bonding network by changing substrate atoms such as N1, N7, or the exocyclic N6 group of 9-deazaadenine might be expected to affect the binding affinity of Immucillins and DADMe-Immucillins. Replacing the 9-deazaadenine ring of pCIPht-DADMe-ImmA [21] (47 fM inhibitor) with an indole ring to give pCIPht-DADMe-Indole [48] (Fig. 9) reduced the dissociation constant to 214 pm, a factor of 4,550 weaker binding, and eliminated slow-onset binding. MT-ImmH [19], which contains a 6-oxo rather than a 6-amino substituent, does not inhibit at 10 µM (Fig. 6) and thus binds less well than MT-ImmA [12] by at least 10^5-fold.

Although the N3 of MT-DADMe-ImmA [32] does not directly interact with any active site residues, the N3 to C3 substitution in MT-DADMe-3-deaza-ImmA [49] caused a 32,500-fold decrease in binding affinity. A loss of ~6 kcal/mol in binding energy is proposed to reflect the altered bond conjugation in the purine ring with different $p_K^*$ values and decreased H-bond interactions at N1, N6, and N7.

Summary and Conclusions—This report adds to the growing demonstration that a detailed understanding of transition state chemistry is readily applied to the design of transition state analogues. Seventeen transition state analogue inhibitors with dissociation constants of 10^-12 to 10^-14 M are described for MTAN from E. coli. Transition state analogue design is based on the riboxacenabtim ion character of the MTAN transition state. With dissociation constants to 47 femtomolar, the 5’-thio-substituted DADMe-Immucillins analogues are among the most powerful noncovalent enzymatic inhibitors. The best of these, pCIPht-DADMe-ImmA [21], exhibits a $K_i^*/K_i^*$ value of 91 million relative to the substrate S-adenosylhomocysteine. In addition to the 17 analogues with dissociation constants from 47 fM to 2 pm, a second group of 21 transition state analogue inhibitors for E. coli MTAN are described with dissociation constants from 2 pm to 1 nM. These inhibitors are potential antibiotics to interfere with the metabolic pathways involved in methylation, polyamine biosynthesis, methionine recycling, and quorum sensing pathways.
Femtomolar Inhibitors of MTAN

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