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Transition-State Analogues of *Campylobacter jejuni* 5'-Methylthioadenosine Nucleosidase

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Abstract

Campylobacter jejuni is a Gram-negative bacterium responsible for food-borne gastroenteritis and associated with Guillain-Barré, Reiter, and irritable bowel syndromes. Antibiotic resistance in *C. jejuni* is common, creating a need for antibiotics with novel mechanisms of action. Menaquinone biosynthesis in *C. jejuni* uses the rare futasine pathway, where 5'-methylthioadenosine nucleosidase (C_jMTAN) is proposed to catalyze the essential hydrolysis of adenine from 6-amino-6-deoxyfutasine to form dehypoxanthinylfutasine, a menaquinone precursor. The substrate specificity of C_jMTAN is demonstrated to include 6-amino-6-deoxyfutasine, 5'-methylthioadenosine, S-adenosylhomocysteine, adenosine, and 5'-deoxyadenosine. These activities span the catalytic specificities for the role of bacterial MTANs in menaquinone synthesis, quorum sensing, and S-adenosylmethionine recycling. We determined inhibition constants for potential transition-state analogues of C_jMTAN. The best of these compounds have picomolar dissociation constants and were slow-onset tight-binding inhibitors. The most potent C_jMTAN transition-state analogue inhibitors inhibited *C. jejuni* growth in culture at low micromolar concentrations, similar to gentamicin. The crystal structure of apoenzyme *C. jejuni* MTAN was solved at 1.25 Å, and five C_jMTAN complexes with transition-state analogues were solved at 1.42 to 1.95 Å resolution. Inhibitor binding induces a loop movement to create a closed catalytic site with Asp196 and Ile152 providing purine leaving group activation and Arg192 and Glu12

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Author Contributions

R.G.D. produced C_jMTAN and kinetically characterized substrates and inhibitions and provided cell-based assays. R.K.H. conducted the structure determinations and characterizations. S.A.C. solved the structure of the C_jMTAN-MTDIA complex. P.C.T. and G.B.E. provided the inhibitors. V.L.S. designed and supervised the project. R.G.D., R.K.H., and V.L.S. wrote the paper. All the authors discussed the data and edited the manuscript.

Supporting Information

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Figures S1–S6; Michaelis–Menten kinetic activity, a structure-based sequence alignment, omit maps for transition-state analogue inhibitors, a stereoview of superimposed inhibitors at the catalytic sites, a chemical diagram of the coupled assay method, and a growth curve for cultured *C. jejuni*; Tables S1 and S2 provide a contact list for the hydrophobic binding pocket contacts and conditions for crystallization and crystal handling (PDF)

The authors declare no competing financial interest.

activating the water nucleophile. With inhibitors bound, the interactions of the 4'-alkylthio or 4'-alkyl groups of this inhibitor family differ from the *Escherichia coli* MTAN structure by altered protein interactions near the hydrophobic pocket that stabilizes 4'-substituents of transition-state analogues. These C_jMTAN inhibitors have potential as specific antibiotic candidates against *C. jejuni*.

Graphical Abstract

Campylobacter jejuni is the most common cause of bacterial-mediated diarrhea. This Gram-negative bacterium has also been implicated as a causative agent of the debilitating paralysis associated with Guillain-Barré syndrome.¹ This pathogen resides in the intestines of farmed animals including poultry and cattle and is transmitted to humans mainly through the consumption of contaminated food.^{2–4} The treatment of *C. jejuni*-mediated gastroenteritis is increasingly more difficult due to the development of drug-resistant strains against common antibiotics, including fluoroquinolones, macrolides, β -lactams, and tetracycline.^{5–7} There is a need for new antibiotics with novel targets against *C. jejuni*.

Bacterial 5'-methylthioadenosine nucleosidase (MTAN) enzymes hydrolyze 5'-methylthioadenosine (MTA), 5'-deoxyadenosine (dAdo), and S-5'-adenosyl-L-homocysteine (SAH) into adenine and S-methyl-5'-thioribose, 5'-deoxyribose, and S-ribosyl-L-homocysteine, respectively.^{8,9} MTANs are involved in adenine and methionine recycling, polyamine synthesis, and quorum sensing in bacteria and are not found in mammals.^{10–16}

The protein folds of the MTANs belong to the MtnN subfamily of the purine nucleoside phosphorylase (PNP)/uridine phosphorylase (UDP) family of enzymes.^{17–19} Recently, it has been reported that *C. jejuni* and *Helicobacter pylori* have unique menaquinone biosynthesis pathways in which MTAN plays an essential role in the hydrolysis of 6-amino-6-deoxyfutasolone (Figure 1). Disrupting menaquinone biosynthesis pathways by blocking MTAN activity is lethal to *H. pylori*.^{20–22} Here, we extend MTAN inhibitor characterization to C_jMTAN and evaluate its target potential by the MTAN inhibitors.

Transition-state analogue inhibitors bind tightly to their cognate enzymes by virtue of their ability to mimic the geometry and electronic environment of an enzymatic transition state. Detailed information on the transition state provides a blueprint to design transition-state analogue inhibitors.^{23–26} These compounds can bind to enzymes much tighter than their natural substrates and therefore are antimicrobial candidates with appropriate targets. Transition-state analogue inhibitors designed for other bacterial MTANs exhibit dissociation

constants to the femtomolar range.^{22,27–30} MTANs catalyze the hydrolysis of the N-ribosidic bond between N9 and C1' of its substrates, via an S_N1 mechanism. Protonation at N7 of the purine leaving group facilitates formation of the transition state. An enzyme-bound water molecule serves as the nucleophile following formation of the ribocationic transition state.^{17,31,32}

RESULTS AND DISCUSSION

Recombinant CjMTAN Expression and Purification.

Expression of recombinant CjMTAN was achieved at 16 h of cell growth in LB medium after IPTG induction of *Escherichia coli* One Shot BL21(DE3) cells harboring a pJ411-CjMTAN construct. Native CjMTAN was expressed with an N-terminal six-histidine tag and purified using Ni-NTA affinity chromatography. The enzyme appeared homogeneous based on SDS-PAGE analysis and yielded 315 mg of active protein from a 2 L culture, and 7.6 g of cell pellet. Experimental details are provided in the methods.

CjMTAN Substrate Specificity.

Most bacterial species use MTANs to recycle MTA, dAdo, and SAH to the adenylate pool and the modified ribosyl groups for methionine synthesis, quorum sensing in the formation of S-ribosylhomocysteine from SAH, or dAdo removal to prevent product inhibition of radical SAM pathways.¹² For the relatively few species using the futasine pathway of menaquinone synthesis, the substrate specificity must also include hydrolysis. *C. jejuni* is one such species, we tested the substrate specificity of CjMTAN using each of these potential substrates, including adenosine (Table 1). CjMTAN exhibits a broad substrate specificity consistent with a role in all of the reactions cited above, including a function in the futasine pathway for menaquinone synthesis. The steady-state kinetic parameters for CjMTAN with MTA and 6-amino-6-deoxyfutasine substrates fit well to a Michaelis–Menten hyperbolic function (Figure S1). However, the substrates SAH, adenosine, and dAdo gave sigmoidal responses to substrate saturation with Hill coefficients of approximately 2 for this dimeric enzyme. MTA is the most active substrate with a $k_{\text{cat}}/K_{\text{m}} = 11.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. Significantly, 6-amino-6-deoxyfutasine was also an active substrate with a $k_{\text{cat}}/K_{\text{m}} = 7.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. SAH, dAdo, and adenosine were also similar, with $k_{\text{cat}}/K_{\text{m}}$ values of 4 to $5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$.

CjMTAN Inhibition by Transition-State Analogues.

Experimentally determined dissociation constants for tightbinding competitive inhibitors were determined in the presence of high substrate concentrations ($141 \times$ the K_{m} value) to shift the apparent K_{i} values to a concentration range permitting $[I] \gg [E]$.²² Transition-state analogue inhibitors causing slow-onset inhibition gave an initial rate followed by a constant reaction rate following slow-onset equilibration. The values of K_{i} were determined from the initial rates, and the values of K_{i}^* were determined from the time-equilibrated rates (Table 2 and Figure 2).

Compounds with three distinct chemical scaffolds were tested for their potential as transition-state analogue inhibitors. HTDIA and BTDIA were picomolar inhibitors at 0.65

and 0.75 nM, respectively. Both have the 5'-thio-DADMe-ImmA scaffold with n-hexyl or n-butyl 5'-thio-substituents. Ten compounds gave dissociation constants in the 1.4 to 6.2 nM range. They include three distinct chemical scaffolds and varied hydrophobicity in the 4'-substituent, including 5'-thio and 4'-desthio structures. Above 50 nM, none of the compounds showed slow-onset inhibition, a characteristic only for the more tightly bound analogues.

The same K_d values of 2.9 nM for MTDIA and EDIA indicate equivalent interaction of the 5'-thio ether and the methylene groups. Comparison of MTDIA (2.9 nM) and MTIA (14 nM) or pCPTDIA (3.1 nM) with pCPTIA (28 nM) indicated a catalytic site preference for the methylene-bridged 3'-hydroxypyrrolidine chemical scaffold, shown to more closely resemble a late S_N1 -like transition state.³⁰ The best inhibitors (HTDIA and BTDIA) have similar K_d values with hydrophobic side chains of eight and six atoms from the 4'-pyrrolidine position. A nine-atom substituent decreases affinity 3-fold in HT6DIA and decreases by 9-fold when the hydroxypyrrolidine ring is opened to form the more flexible HSMIA. The hydrophobic character of the 4'-substituent binding pocket on the enzyme is revealed in PETDIA, whose eight-atom 4'-substituent causes a 5-fold decrease in affinity relative to HTDIA because of an oxygen ether and a primary alcohol in the substituent. When three oxygens are in a nine-atom linear 4'-substituent (dPDIA), the dissociation constant increased to 1.7 μ M. Linear hydrophobic 4'-substituents are favored, as bulky hydrophobic groups increase dissociation constants to 19 and 422 nM, respectively, in the adamant- and naphthyl-substituted compounds, ATDIA and NIA. The critical nature of a hydrophobic group as the 4'-substituent is emphasized by the nearly 1000-fold difference in affinity between MTDIA (2.9 nM) and DIA (2.7 μ M).

Although *C. jejuni* and *H. pylori* both use the futasoline pathway for synthesis of menaquinone, the MTANs of *C. jejuni* and *H. pylori* (HpMTAN) share only 50% identity and 67% similarity. Inhibition studies with HpMTAN,²² compared to the values for CjMTAN, indicate tighter binding to HpMTAN. For example, the four best inhibitors for CjMTAN gave K_d values from 0.7 to 1.4 nM. The same four inhibitors for HpMTAN gave dissociation constants of 5 to 41 pM.²² The structural basis for these differences are discussed below.

CjMTAN Inhibitors Inhibit Cell Growth.

Five inhibitors displaying the highest affinity for CjMTAN were measured on *C. jejuni* growth in Mueller-Hinton broth. The half-maximum inhibitory concentrations (IC_{50}) for HexS-DADMe-ImmA (HTDIA), BuS-DADMe-ImmA (BTDIA), 2-pyrazineSDADMe-ImmA (PTDIA), 5'-deoxy-5'-Pro-DADMe-ImmA (PDIA), and MeS-DADMe-ImmA (MTDIA) were $1.3 \pm 0.3 \mu$ M, $1.4 \pm 0.3 \mu$ M, $2.9 \pm 0.9 \mu$ M, $3.1 \pm 0.2 \mu$ M, and $4.2 \pm 1.3 \mu$ M, respectively. Gentamicin, used as a control, had an IC_{50} value of $2.1 \pm 0.1 \mu$ M, making the CjMTAN inhibitors approximately equal to a clinically effective antibiotic (Figure 3, panel A).

Cell growth assays were also performed on solid Mueller-Hinton agar to determine the effect of HTDIA, BTDIA, PTDIA, PDIA, MTDIA, (S)-Hex-SerMe-ImmA (HSMIA), and Me-SSerMe-ImmA (MTSMIA). *C. jejuni* susceptibility to these compounds was determined by

monitoring colony formation over the course of six consecutive days to establish IC₅₀ values. All compounds tested in culture inhibited bacterial growth (Figure 3, panel B), with IC₅₀ values in the low micromolar range. IC₅₀ values were HTDIA = $1.3 \pm 0.7 \mu\text{M}$, BTDIA = $1.5 \pm 0.8 \mu\text{M}$, PTDIA = $3.0 \pm 0.7 \mu\text{M}$, PDIA = $3.1 \pm 0.7 \mu\text{M}$, MTDIA = $3.5 \pm 0.7 \mu\text{M}$, HSMIA = $5.6 \pm 0.8 \mu\text{M}$, and MTSMIA = $135.4 \pm 2.8 \mu\text{M}$. Gentamicin, used as a control, had an IC₅₀ value of $0.39 \pm 0.06 \mu\text{M}$. These IC₅₀ values are similar to those for inhibition of *C. jejuni* growth in liquid media.

CjMTAN Structure and Comparisons with other MTANs.

The unliganded and five inhibitor bound structures of CjMTAN were solved with resolutions from 1.25 to 1.95 Å in different space groups (Table 3). All refined structures have acceptable R/R_{free} scores. Structures provided well-visualized electron density maps except for the N-terminal His6-tag purification sequence and a few side chain residues at the surface of the protein. Unliganded CjMTAN was solved at 1.25 Å resolution in space group $P2_12_12_1$ with two monomers (a dimer) in the asymmetric unit. The PISA server analysis suggested the dimer of the asymmetric unit represents the physiological oligomeric state of CjMTAN.³³ The solvent-accessible surface area of the dimer interface is 1424 Å². The structural fold of CjMTAN is similar to previously described MTAN structures with seven helices including one 3₁₀-helix and 10 β-sheets.^{17,19} These are arranged in three αβα-layer structures with central mixed β-sheets (Figures 4 and S2).

Ligand-induced active site closing can be compared to the HpMTAN and *Salmonella enterica* MTAN (SeMTAN) structures.^{18,19} Ligand binding to one monomer induces negative cooperativity to form one ligand-occupied active site in a closed conformation, and a ligand-free second active site. Unliganded CjMTAN gave both active sites in the open conformation. The RMSD of CjMTAN when one subunit (chain-B) is superimposed on the other (chain-A) is 0.305 Å, thus, the Ca's of both chains are nearly identical. When other apo-MTAN structures have been analyzed, most have one monomer bound to a copurifying ligand (adenine) with a closed active site conformation. Unliganded CjMTAN is unusual with both active sites in open conformations and no copurifying ligand. Only unliganded *E. coli* MTAN (EcMTAN, PDB ID: 1Z5P) also presents both monomers with open conformations.³⁴

Catalytic Site of CjMTAN.

The catalytic site of CjMTAN is buried in the structure by a major structural rearrangement involving conversion of an unstructured loop into an extension of the α6 helix when inhibitors bind the catalytic site (Figure 5).

The RMSE comparison of unliganded and five inhibitor bound structures varies from 0.979 to 1.041 Å. In the inhibitor bound structures, there are conformational changes seen in the peptides from Asp196 to Val221, Gly148 to Lys158, and Leu100 to Val112 (Figures 4 and 5). The largest conformational changes are the Ala202 Ca and Asp196 carboxylate atoms which move by 7.3 and 5.1 Å, respectively, on active site closing. These conformational changes are similar to those previously described for *E. coli* and *H. pylori* MTANs.^{17–19}

A catalytic site loop from residues Gly7 to Thr14 has a small shift to cause a 2.9 Å movement in the carboxylate carbon of Glu12, responsible for activation of the nucleophilic water prior to its attack on the C1' of the substrate (Figure 6). In the unliganded structure, Glu12 is far from the active site. In the inhibitor bound structure, the Glu12 carboxyl atom forms a hydrogen bond with the active site water (Figure 5). Inhibitor binding positions the β 8- α 4 loop to place the side-chain of Phe151 in a hydrophobic stacking interaction with adenine. The peptide amino and carbonyl groups of Ile152 form hydrogen bonds with the purine leaving group. Asp196 stabilizes the N7 proton of the 9-deazaadenine, also a feature of the transition state. Asp196 rotates 180° opposite to the active site with an open catalytic site and is reported to be important for N7 protonation at the transition state.^{31,34}

DADMe-ImmA Analogue Binding.

Crystal structures of C₇MTAN with five transition-state analogue inhibitors gave well-defined electron density maps (Table 3, Figure S3). These inhibitors mimic a late dissociative transition state, but with different 4'-substituents on the 3'-hydroxypyrrolidine group (Figures 6 and S4). The inhibitors PDIA, MTDIA, BTDIA, HTDIA, and PTDIA filled both active sites of C₇MTAN to form closed conformations (Figure 5).

All complexes have a carboxylate oxygen of the Asp196 hydrogen bonded to N7 of the adenine, a transition state feature. Distances from the Asp196 oxygen to the N7 (O–N distances) are 2.7 to 2.8 Å. The second carboxylate oxygen of Asp196 accepts a hydrogen bond from the N6-amino group of adenine with the O–N distances of 2.9 to 3.0 Å. The N1 and N6 of adenine share hydrogen bonds with backbone nitrogen and oxygen atoms of Ile152 with distances of 2.7 to 2.9 Å. The N1' cation of the inhibitor, corresponding to the C1'-ribocation of the substrate, is 2.7 to 2.8 Å from the nucleophilic waters, also hydrogen bonded to Glu12 and Arg192 (2.6 to 2.8 Å). The 3'-hydroxyl group of the pyrrolidine is hydrogen bonded with the carboxylate of Glu173 (2.6 to 2.7 Å; Figure 6). The geometry of the bound methylhydroxypyrrolidine moieties of the inhibitors is the same within crystallographic limits.

Purine binding to C₇MTAN is the same as reported for *H. pylori* and *E. coli* MTANs. Differences in the inhibitor binding mode for C₇MTAN appear in the 4'-alkyl and 4'-alkylthio group binding pocket.

Structure-Inhibition Relationship of Transition-State Analogues.

Inhibition experiments with C₇MTAN gave dissociation constants of 0.65 to 2.9 nM for HTDIA, BTDIA, PTDIA, PDIA, and MTDIA (Table 2). HTDIA and BTDIA are slow-onset tight-binding inhibitors with a K_i^* of 0.65 nM and 0.75 nM, respectively. The hydrophobic 4'-alkylthio binding site is formed at the dimer interfaces by Phe151(A), Phe206(A), Ile102(B), Met9(A), Phe105(B), Ile50(A), His107(B), Phe111(B), and Pro113(B) with contributions from both the A and B monomers. The flexible, hydrophobic 4'-substituent binding site prefers linear hydrophobic groups of three to nine atoms, exemplified by the 4'-CH₂S(CH₂)₅CH₃ of HTDIA (0.65 nM), 4'-CH₂S(CH₂)₃CH₃ of BTDIA (0.75 nM), 4'-(CH₂)₃CH₃ of PDIA (1.4 nM), 4'-(CH₂)₂S(CH₂)₅CH₃ of HT6DIA (2.0 nM), and 4'-(CH₂)₂CH₃ of EDIA (2.9 nM). The flexibility of the site also accepts more bulky

substituents including 4'-methylthio-2-pyrazine (PTDIA, 1.4 nM) and 4'-methylthiocyclohexane (CHTDIA, 2.2 nM). These specificities are consistent with the biological function proposed for C₇MTAN, where the natural substrates include MTA, dAdo, SAH, and 6-amino-6-deoxyfutasine.¹² Thus, the most effective inhibitor for C₇MTAN was HTDIA with a dissociation constant of 0.65 nM, binding 11 000 times more tightly than MTA, also the most active substrate. In another example, HCTDIA, the transition-state analogue of SAH, gives a dissociation constant of 24 nM, binding 500-fold more tightly than the apparent K_m for SAH.

In the C₇MTAN-MTDIA complex, the short 5'-methylthio group is completely buried in the active site with contacts to Ile50 (chain-A) and Phe105 (chain-B). The remaining space in the hydrophobic 4'-binding tunnel is partly filled with a glycerol molecule in hydrogen bond interactions with a structural water (Wat126), His107 (B), and Asp207 (A). Active site contacts to the 4'-groups of transition-state analogues can be compared (Table S1). The 4'-hexylthio group in the C₇MTAN-HTDIA complex has the highest number of interactions and is the best inhibitor. The terminal methyl group of the hexyl group in HTDIA is near the exit of the binding pocket, in contact with solvent.

Bacterial MTANs show remarkable species-specific affinity for transition-state analogues.^{11,16} For example, the dissociation constants for MTDIA for the MTANs from *E. coli*, *N. meningitidis*, *H. pylori*, *K. pneumoniae*, *S. aureus*, *S. pneumoniae*, and *C. jejuni* are 2, 140, 571, 784, 1400, 24 000, and 2900 pM, respectively, to span a factor of 12 000 in affinity. One explanation for these differences includes altered whole-protein dynamic motions in the MTANs leading to different entropic contributions to binding.^{35,36} Local dynamic differences are also evident in B-factors at the protein surface surrounding the hydrophobic pockets. Thus, the B-factors for the loops covering the hydrophobic binding site suggest a more stable, closed protein structure at the catalytic site region in EcMTAN than in the C₇MTAN (Figure 7).

CONCLUSIONS

C. jejuni is responsible for the most common food-borne gastrointestinal disorders including diarrhea. C₇MTAN catalyzes an essential reaction in the synthesis of menaquinone, where 6-amino-6-deoxyfutasine is converted into dehypoxanthinylfutasine in the *C. jejuni* pathway. MTAN has been reported to be essential in *H. pylori*, which belongs to the same bacterial order as *Campylobacterales*. Transition-state analogues of MTAN reactions were effective in C₇MTAN inhibition with binding dissociation constants in the picomolar or low nanomolar range. Some of the most potent compounds showed *C. jejuni* antibacterial activity with IC₅₀ values in the low micromolar range similar to known antibiotics. As only a few species use the futasine pathway, the inhibitors described here are anticipated to have minimal effects on the gut microbiome. Crystal structures of unliganded C₇MTAN are compared with complexes of five transition-state analogue inhibitors. The nature of open and closed catalytic sites revealed a common binding mode for the 9-deazaadenine groups of the inhibitors but unique binding modes for hydrophobic groups 4'- to the 3'-hydroxypyrrolidine mimic of the proposed ribocationic transition state. The most effective inhibitors for C₇MTAN also provided the lowest IC₅₀ for inhibition of bacterial growth,

supporting the hypothesis of CjMTAN inhibition as a potential antibiotic strategy against *C. jejuni*.

METHODS

Chemicals.

The synthesis and characterization of all transition-state analogue inhibitors has been reported elsewhere, as follows: HexSDADMe-ImmA (HTDIA), 2-PyrazineS-DADMe-ImmA (PTDIA), (S)-Hex-SerMe-ImmA (HSMIA), HT6DIA, PETDIA, ATDIA, FDIA, PTSMIA, dPDIA, FIA,²² BuS-DADMe-ImmA (BTDIA), MeS-DADMe-ImmA (MTDIA), CHTDIA, EDIA, pCPTDIA, PhTDIA,³⁷ 5'-deoxy-5'-Pro-DADMe-ImmA (PDIA), MDIA,²⁹ Me-SSerMe-ImmA (MTSMIA),²⁸ MTIA, pCPTIA, NIA,³⁸ HCTDIA, dDIA,³⁰ DIA,³⁹ IAMS,⁴⁰ IA,⁴¹ and MTT.⁴² All transition-state analogue inhibitors of CjMTAN were dissolved in sterile 50 mM Hepes-KOH at pH 7.4 and had their concentration estimated based on the extinction coefficient ($8.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 275 nm). These stock solutions were further diluted with culture media prior to addition into the corresponding cultures (media and/or agar plates) in the antibacterial activity assays. All other chemicals used were of analytical or reagent grade, or were of the highest purity commercially available, and were not subject to additional purification.

Recombinant Enzyme Expression and Purification.

The codon-optimized open reading frame for production of native CjMTAN (*Campylobacter jejuni* subsp. *jejuni* strain 81-176; UniProt ID A0A0H3PEB1) in *E. coli* was purchased from ATUM and inserted into pJ411, an inducible high-level expression plasmid. An N-terminal six-histidine tag was added to assist subsequent protein purification steps. Nucleotide sequencing was performed to validate the DNA sequence for CjMTAN. The pJ411-CjMTAN construct was transformed into *E. coli* One Shot BL21(DE3) transformation-competent cells (Invitrogen) and plated. A single colony from overnight culture was grown in LB with kanamycin ($50 \mu\text{g mL}^{-1}$) medium at 37 °C and 180 rpm to an optical density of 0.6–0.9 absorbance units ($\lambda = 600 \text{ nm}$). Protein expression was induced by 350 μM IPTG at 25 °C. After 16 h, cells were harvested by centrifugation (5000g for 20 min) and stored at –80 °C. All subsequent steps were performed at 4 °C, unless stated otherwise.

Cells were suspended in 20 mM KH_2PO_4 –KOH, 500 mM KCl, 5 mM imidazole, and 1 mM DTT (pH 7.0; 6.5 mL/g of cell pellet), together with a few milligrams of lysozyme, DNaseI (Sigma), and protease inhibitor cComplete Mini EDTA-free (one tablet per 7.6 g of cell pellet; Roche). After being stirred for 30 min, the cells were disrupted by sonication (15 s on, 15 s off, at 30% amplitude for 30 min) and centrifuged (20 000g for 20 min) to remove cell debris. The supernatant was incubated with Ni-NTA agarose (1.7 mL of slurry/g of cell pellet; Qiagen) for 45 min with rocking, and the mixture was poured into a column and washed with 13 column volumes of the cell suspension buffer. The collection of five-column-volume fractions from a 50–500 mM imidazole stepwise elution gradient was followed by SDS-PAGE (200 V 185 mA for 60 min in MOPS running buffer) analysis, and the fractions containing the target protein with purity over 95% were pooled and dialyzed against 20 mM KH_2PO_4 –KOH, 100 mM KCl, 1 mM DTT, and 10% glycerol (pH 7.0) using

10 kDa dialysis cassettes (Thermo Scientific). The enzyme solution was concentrated to approximately 380 μM (10 mg mL⁻¹, extinction coefficient estimated to be 10 430 M⁻¹ cm⁻¹ at 280 nm). Small aliquots were frozen in liquid nitrogen and stored at -80 °C.

Determination of Kinetic and Inhibition Constants.

Steady-state kinetic parameters and inhibition constants for the C_JMTAN reaction were determined under initial rate conditions at 25 °C in 50 mM Hepes-KOH at pH 7.4 (1 mL total reaction volumes). C_JMTAN catalytic activity was monitored in a Cary 300 spectrophotometer (Varian) with 1 cm path length cuvettes using an absorbance-based coupled enzyme assay (Figure S5). Given the small spectrophotometric change following the hydrolysis of MTA to adenine (and methylthioribose), xanthine oxidase (Sigma) was added to the reaction mixtures to convert adenine into 2,8-dihydroxyadenine, which gives a significant time-dependent increase in absorbance at 305 nm, where the absorption coefficient for 2,8-dihydroxyadenine is 15.5 mM⁻¹ cm⁻¹. All substrate specificity compounds were dissolved in 50 mM Hepes-KOH at pH 7.4, and their concentrations were estimated based on the extinction coefficient at 260 nm (15.9 mM⁻¹ cm⁻¹).

Steady-state kinetic parameters and catalytic efficiencies (k_{cat} , K_{m} , and $k_{\text{cat}}/K_{\text{m}}$) for C_JMTAN were determined in assays containing varying concentrations of MTA, 6-amino-6-deoxyfutasoline, SAH, dAdo, or adenosine ranging from 1 to 100 μM and 1 U xanthine oxidase and were initiated with 50 nM C_JMTAN. Reaction mixtures were monitored for up to 5 min, and the substrate saturation curves were fitted to Michaelis–Menten hyperbolic (eq 1) and/or allosteric sigmoidal (eq 2) functions. Initial rates were the average of duplicate or triplicate measurements. The corresponding values were used for statistical analysis using GraphPad Prism 6.07.

$$v = VA/(K_a + A) \quad (1)$$

$$v = VA^h/(K_{\text{half}} + A^h) \quad (2)$$

For eqs 1 and 2, v is the measured reaction rate, V is the maximal rate, A is the substrate concentration, K_a and K_{half} report on the Michaelis constant, and h is the Hill coefficient.

The equilibrium inhibition constants (K_i^*) for C_JMTAN with the transition-state analogue inhibitors were determined in assays containing MTA at 1 mM, 1 U xanthine oxidase, an inhibitor at concentrations from 3 nM to 100 μM , and were initiated by the addition of 50 nM C_JMTAN. Reaction mixtures were monitored for up to 2 h to permit development of slow-onset binding. Inhibitor-free mixtures with and without addition of C_JMTAN were used as control reactions. The reaction rates obtained with each transition-state analogue inhibitor in the reaction mixtures were fitted to the Morrison enzyme inhibition kinetics equation for slow-onset, tight-binding inhibitors (eq 3).⁴³ Initial rates were the average of duplicate or

triplicate measurements. The corresponding values were used for statistical analysis using GraphPad Prism 6.07.

$$v/v_0 = (K_a + A)/(K_a + A + K_a I/K_i) \quad (3)$$

For eq 3, v and v_0 are the initial velocities with and without inhibitor, I is the inhibitor concentration, and K_i is the equilibrium inhibition constant.

***C. jejuni* Culture Conditions.**

Culture media for *C. jejuni* was prepared every week, sterilized, and stored at room temperature. Each liter (distilled water) contained 21 g of Mueller-Hinton broth (Oxoid), and the final solution was sterilized by autoclaving at 121 °C for 15 min. The pH was 7.0. Mueller-Hinton Agar was made according to the manufacturer (Difco), sterilized by autoclaving at 121 °C for 15 min, poured into flat bottom tissue culture treated six-well plates (CytoOne), and stored at 4 °C. Although many types of media can be used to culture *C. jejuni*, Mueller-Hinton is reported to have the highest recovery rate.⁴⁴ Culture media and agar plates (solid media) were warmed to 42 °C before each use.

Freeze-dried *C. jejuni* strain AS-83–79 was purchased from ATCC (item number 33560) and stored at 4 °C. The entire pellet was hydrated with 1.5 mL of culture media and aseptically transferred to a 75 cm² blue plug seal cap tissue culture treated flask (Falcon) containing 25 mL of culture media. *C. jejuni* was subject to routine *in vitro* culturing at 42 °C under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂ gas composition). The culture media were changed daily, and a Belly Button mini-orbital shaker (Sigma) was used to maintain cultures under constant agitation. The optical density was monitored daily and kept below 1.5 absorbance units (λ = 600 nm). Since a significant loss of viable bacteria can occur when *C. jejuni* are left at RT and ambient atmosphere,⁴⁵ the time that cultures were out of the microaerophilic atmosphere was kept to a minimum.

Exponential Cell Growth.

The doubling time for *C. jejuni* in liquid culture was determined in culture flasks containing 25 mL of culture media, inoculated with 200 μ L of an overnight *C. jejuni* culture displaying an optical density of 0.7 absorbance units (λ = 600 nm). Optical density was measured over 16.4 h, and the corresponding values were used for statistical analysis using GraphPad Prism 6.07. Under these conditions, *C. jejuni* displayed a doubling time of 2 h 28 min ($\pm$ 5 min; Figure S6).

IC₅₀ Assays in Culture Media.

The effect of HexS-DADMe-ImmA (HTDIA), BuS-DADMe-ImmA (BTDIA), 2-pyrazines-DADMe-ImmA (PTDIA), 5'-deoxy-5'-Pro-DADMe-ImmA (PDIA), and MeSDADMe-ImmA (MTDIA) on *C. jejuni* growth was used to establish IC values using liquid culture media.⁴⁶ In brief, *C. jejuni* cultures (200 μ L/well in a 96-well plates) with an initial optical density of 0.005 absorbance units (λ = 600 nm) were grown in the presence of increasing

concentrations of each compound. The transition-state analogue inhibitor concentrations were kept at a series of 3-fold dilutions, ranging from 1 nM to 20 μ M. Wells containing no inhibitor served as controls for their respective plates. Gentamicin was used as a positive control, at concentrations ranging from 500 nM to 10 mM. All culture media flasks were kept in a sealed gas chamber. *C. jejuni* susceptibility to these compounds was determined after 16.4 h in culture by measuring the cell growth based on optical density (600 nm) using a SpectraMax M5 microplate reader (Molecular Devices). Statistical analyses to determine the IC₅₀ values were performed with GraphPad Prism 6.07 using a nonlinear regression curve fitting.

IC₅₀ Inhibition Assays in Agar Media.

The effects of Hex-SDADMe-ImmA (HTDIA), Bu-S-DADMe-ImmA (BTDIA), 2-Pyrazine-S-DADMe-ImmA (PTDIA), 5'-deoxy-5'-Pro-DADMe-ImmA (PDIA), Me-S-DADMe-ImmA (MTDIA), (S)-Hex-SerMe-ImmA (HSMIA), and Me-S-SerMe-ImmA (MTSMIA) were evaluated on *C. jejuni* growth in an agar assay.²² In brief, *C. jejuni* agar media cultures (5 mL/well in a six-well plates) containing 25 μ L of overnight culture at an optical density of 0.5 absorbance units (λ = 600 nm) were grown in the presence of increasing concentrations of each compound. Sterile glass beads were used to evenly spread inoculum into the wells. The transition-state analogue inhibitor concentrations were 6 nM to 60 μ M, except for MTSMIA, with concentrations from 43 nM to 430 μ M. Wells containing no inhibitor served as negative controls. Gentamicin was tested at concentrations ranging from 40 nM to 400 μ M and served as a positive control. All agar media cultures were kept in a sealed gas chamber. *C. jejuni* susceptibility to these compounds was determined by monitoring colony number over the course of 6 days. Statistical analyses to determine the IC₅₀ values were performed with GraphPad Prism 6.07 using a nonlinear regression curve fitting.

Crystallization.

Crystallization experiments with CjMTAN used sitting drop vapor diffusion at 22 °C. Protein (10 mg mL⁻¹) was screened with the Microlytic (MCSG1–4) and Hampton (crystal screenHT) crystallization conditions. Crystallization drops were set up in 96-well INTELLI plates (ART ROBBINS) using the CRYSTAL-GRYPHON crystallization robot (ART ROBBINS). Each crystallization drop contained 0.5 μ L of CjMTAN and 0.5 μ L of well solution. The volume of the well solution was 70 μ L. Good quality crystals were obtained in 1 week.

Co-crystallization of CjMTAN with transition-state analogue inhibitors (PDIA, MTDIA, BTDIA, HTDIA, and PTDIA) used 10 mg mL⁻¹ CjMTAN mixed with inhibitors in a 1:2 molar ratio and incubated for 2 h on ice. The crystallization experiment was done as described above for unliganded enzymes. The crystallization and crystal handling process is summarized in Table S2.

Data Collection and Data Processing.

Diffraction data from unliganded and inhibitor-bound CjMTAN crystals were collected from 1.25 to 1.95 Å resolution (Table 3) at the LRL-CAT beamline (Argonne National Laboratory, Argonne, IL) irradiated at 0.97931 Å wavelength. Diffraction data were

processed using the iMOSFLM⁴⁷ protocol and scaled by the AIMLESS program of the CCP4 suite⁴⁸ in different space groups as summarized in Table 3. The quality of the diffraction data was analyzed in the SFCHECK and XTRIAGE programs.^{48,49} The Matthews coefficient (V_m) calculations were done to calculate monomers present in the unit cells. The data collection and processing statistics are summarized in Table 3.

Structure Determination and Refinement.

Crystal structures of C₇MTAN, unliganded, and complexed with transition-state analogue inhibitors were solved by molecular replacement using PHASER.⁵⁰ Chain-A of wild-type unliganded HpMTAN (PDB ID: 3NM4) was used as the initial phasing model. The model obtained from PHASER was manually adjusted and completed using the graphics program COOT.⁵¹ The structure refinement was performed by the REFMAC5 program, using standard protocols for the NCS refinement.⁵² The inhibitor molecules were left out of the models to initiate the refinement. After including water into the structures, inhibitor molecules were fitted in their respective electron densities. The final refinement statistics of the structures are summarized in Table 3.

Structure Analysis.

The crystal structures of unliganded HpMTAN (PDB ID: 3NM4, chain: A) and EcMTAN complexed with adenine (PDB ID: 1JYS, chain: A) have been used for structure comparisons. The BTDIA complex with HpMTAN (PDB ID: 4WKO, chain: A) and EcMTAN (PDB ID: 4WKC, chain: A) have also been used in the structural comparisons. Structural superimpositions used the SSM protocol of COOT. The geometry analyses of the final model used MolProbity.⁵³ Further structure analyses, including the calculation of the B-factor profiles, used the BAVEGAGE program of the CCP4 suite.⁴⁸ Structural figures were produced in the molecular graphics program PyMOL. For C₇MTAN structures, subunit-A was used for all the structural analyses and comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
CjMTAN catalyzes the hydrolysis of 6-amino-6-deoxyfutasine in the menaquinone biosynthesis pathway.

Figure 2.

Transition-state analogues tested against *Cj*MTAN. Compounds are ranked based on K_i^* and K_i values (Table 2). Above 50 nM, none of the compounds show slow-onset inhibition, a characteristic reserved for more tightly bound analogues. These are ranked on K_i values, in parentheses.

Figure 3.
Half-maximum inhibitory concentration (IC₅₀) for *C. jejuni* grown in (A) Mueller-Hinton Broth and (B) Mueller-Hinton Agar with a set of transition-state analogue inhibitors.

Figure 4.

(A) Quaternary homodimeric structure of unliganded CjMTAN. The mode of dimerization is mediated by $\alpha 2$ and shown by the black arrow. The active site of the enzyme is also highlighted. The loop between $\beta 6$ and $\beta 7$ strands of subunit-A extends toward the side of subunit-B (and vice versa). This extended loop participates in the formation of the substrate binding pocket of the neighboring subunits. The catalytic sites are deeply buried in the structure. The active site residues are also highlighted with bound HTDIA. (B) Stereoview of the surface representation of the apo-CjMTAN modeled with HTDIA. The open binding pocket is highlighted. (C) Stereoview of the surface representation of the CjMTAN-HTDIA complex. The closed binding pocket is highlighted. The orientation of the structure is the same as B.

Figure 5.

Conformational changes in the CjMTAN structure upon transition-state analogue binding.

(A) Structural comparison of CjMTAN structures (stereoview) in open (unliganded, PDB ID: 6AYM) and closed (HTDIA bound, PDB ID: 6AYS) conformation. The structure shown in cyan is the unliganded open conformation monomer-A of CjMTAN, and the monomer colored in brick red shows the HTDIA-bound closed form. The labeled regions move and show large structural changes upon ligand binding. (B) The superposition of the binding site of CjMTAN structures (stereoview) in open (unliganded, PDB ID: 6AYM) and closed (HTDIA bound, PDB ID: 6AYS) conformation. Helix 4 and 6 and associated loops, which are important for ligand binding, are highlighted. The conformation changes of the catalytic residue Glu12 (cyan, open form; brick red, closed form) are also shown.

Figure 6.

Stereoview of the binding sites of CjMTAN complex with transition-state analogue inhibitors. The binding of inhibitors PDIA, MTDIA, BT DIA, HT DIA, and PT DIA are shown in panels A, B, C, D, and E, respectively. The residues interacting with inhibitors from monomer-A are shown in green and from monomer-B are shown in light blue. The hydrogen bonding interactions are shown in orange dotted lines.

Figure 7.

Atomic motion expressed as crystallographic B-factors comparing the catalytic sites of EcMTAN and CjMTAN. (A) Stereoview of the surface representation of the binding site of CjMTAN-BTDIA complex. (B) Stereoview of the surface representation of the binding site of EcMTAN-BTDIA complex. The orientation of the structures is the same in both panels. Surface color is red for the most active regions and shaded toward blue for the amino acids with the lowest B-factors.

Table 1.

Substrate Specificity and Kinetic Parameters for C₁MTAN^a

substrate	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (M ⁻¹ s ⁻¹)	h^b
5'-methylthioadenosine	0.80 ± 0.02	7.1 ± 0.7	11.3 (±1.1) × 10 ⁴	
6-amino-6-deoxyfutalosine	0.27 ± 0.01	3.5 ± 0.4	7.8 (±0.9) × 10 ⁴	
S-5'-Adenosyl-L-homocysteine	0.55 ± 0.03	12.2 ± 1.5	4.6 (±0.6) × 10 ⁴	2.6 ± 0.7
5'-deoxyadenosine	0.92 ± 0.02	21.4 ± 1.3	4.3 (±0.3) × 10 ⁴	1.8 ± 0.2
adenosine	0.30 ± 0.01	7.5 ± 0.6	4.0 (±0.3) × 10 ⁴	2.0 ± 0.4

^a At 25 °C and 50 mM Hepes-KOH pH 7.4.

^b Hill coefficient.

Table 2.Inhibition Constants for Transition-State Analogue Inhibitors of C₇MTAN^a

compound	K _i (nM)	K _i [*] (nM)
HTDIA	3.3 ± 0.6	0.65 ± 0.17
BTDIA	4.1 ± 1.0	0.75 ± 0.20
PTDIA	5.7 ± 0.7	1.4 ± 0.2
PDIA	5.1 ± 0.6	1.4 ± 0.1
HT6DIA	4.4 ± 0.6	2.0 ± 0.2
CHTDIA	5.6 ± 0.6	2.2 ± 0.1
EDIA	7.6 ± 0.9	2.9 ± 0.2
MTDIA	6.5 ± 0.6	2.9 ± 0.1
pCPTDIA	4.8 ± 0.3	3.1 ± 0.1
PETDIA	11.0 ± 1.3	3.2 ± 0.3
PhTDIA	5.3 ± 0.3	4.0 ± 0.2
HSMIA	7.0 ± 0.4	6.2 ± 0.3
MDIA	14.3 ± 1.0	10.0 ± 0.4
MTIA	32.3 ± 3.4	13.7 ± 0.6
HCTDIA	45.1 ± 3.4	23.6 ± 1.2
ATDIA	19.1 ± 2.1	
pCPTIA	27.8 ± 3.0	
dDIA	55.3 ± 5.9	
FDIA	106 ± 11	
MTSMIA	216 ± 29	
NIA	422 ± 75	
PTSMIA	946 ± 44	
dPDIA	1652 ± 138	
DIA	2607 ± 332	
FIA	2651 ± 392	
IAMS	12520 ± 5052	
IA	13910 ± 2008	
MTT	22145 ± 6084	

^aFigure 2 for structures.

Table 3.

Data Collection and Refinement Statistics

data set ^a	Campylobacter jejuni 5'-methylthioadenosine nucleosidase (CjMTAN)					
	CjMTAN-Apo	CjMTAN + PDIA	CjMTAN + MTIDIA	CjMTAN + BTIDIA	CjMTAN + HTIDIA	CjMTAN + PTIDIA
unit cell data						
space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁	P2 ₁	P2 ₁	P1	P2 ₁
cell parameters (Å, deg)	<i>a</i> = 46.41, <i>b</i> = 84.62, <i>c</i> = 124.88 <i>a</i> , <i>β</i> , <i>γ</i> = 90	<i>a</i> = 37.36, <i>b</i> = 90.08, <i>c</i> = 67.37 <i>a</i> , <i>γ</i> >= 90 <i>β</i> = 105.5	<i>a</i> = 37.13, <i>b</i> = 90.09, <i>c</i> = 67.83 <i>a</i> , <i>γ</i> = 90 <i>β</i> = 104.6	<i>a</i> = 68.16, <i>b</i> = 91.41, <i>c</i> = 78.43 <i>a</i> , <i>γ</i> = 90 <i>β</i> = 110	<i>a</i> = 67.37, <i>b</i> = 75.29, <i>c</i> = 90.44 <i>a</i> = 87.5, <i>β</i> = 89.1, <i>γ</i> = 71.1 71.1	<i>a</i> = 71.08, <i>b</i> = 90.98, <i>c</i> = 72.45 <i>a</i> , <i>γ</i> = 90 P = 111.4
<i>V_m</i> (Å ³ /Dalton)	2.3	2.1	2.1	2.2	2.1	2.1
number of subunits in the asymmetric unit	2	2	2	4	8	4
data collection						
beamline	LRL-CAT	LRL-CAT	LRL-CAT	LRL-CAT	LRL-CAT	LRL-CAT
wavelength (Å)	0.97931	0.97931	0.97931	0.97931	0.97931	0.97931
temperature (K)	100	100	100	100	100	100
resolution range (Å)	70.05–1.25 (1.27–1.25)	64.92–1.67 (1.70–1.67)	65.65–1.42 (1.45–1.42)	73.72–1.95 (2.00–1.95)	90.35–1.70 (1.73–1.70)	67.44–1.85 (1.89–1.85)
total number of observed reflections	976320 (47597)	372356 (16727)	338217 (17647)	269003 (18687)	750704 (37638)	543600 (33658)
number of unique reflections	134158 (6445)	49714 (2525)	81192 (4310)	65501 (4607)	179940 (8786)	73146 (4499)
<i>R_{merge}</i> (%) ^b	8.3 (110.4)	11.7 (100.0)	5.1 (76.7)	8.4 (84.5)	10.7 (49.2)	10.6 (90.7)
<i>R_{rim}</i> (%) ^c	3.3 (43.1)	4.6 (41.4)	2.9 (43.1)	4.7 (47.6)	6.2 (27.0)	4.2 (35.6)
CC1/2 (%)	99.9 (65.3)	99.7 (72.8)	99.9 (70.6)	99.8 (65.8)	98.9 (83.8)	99.8 (88.5)
<i>⟨I/σ(I)⟩</i> ^d	14.8 (1.9)	11.6 (2.0)	15.4 (2.0)	11.5 (1.9)	8.1 (2.1)	10.2 (1.9)
completeness (%)	98.3 (96.5)	99.7 (97.5)	99.8 (100)	99.4 (99.9)	97.3 (95.7)	99.9 (100)
multiplicity	7.3 (7.4)	7.5 (6.6)	4.2 (4.1)	4.1 (4.1)	4.2 (4.3)	7.4 (7.5)
Wilson B-factor (Å ²) refinement	9.0	15.2	13.1	28.5	13.8	24.6
<i>R_{work}</i> (%) ^e	18.9	18.0	17.2	22.4	23.0	21.9

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Campylobacter jejuni 5'-methylthioadenosine nucleosidase (CjMTAN)						
data set ^a	CjMTAN-Apo	CjMTAN + PDIA	CjMTAN + MTDIA	CjMTAN + BTDIA	CjMTAN + HTDIA	CjMTAN + PTDIA
$R_{\text{free}} (\%)^f$	20.5	20.1	18.5	25.3	24.8	24.5
no. of atoms	4226	3954	4064	7423	16054	7689
protein atoms	3589	3535	3545	7052	14124	7068
ligand atoms		40	40	92	200	100
solvent atoms	637	379	479	279	1730	521
model quality						
RMS deviation from ideal value						
bond length (Å)	0.008	0.007	0.007	0.009	0.007	0.007
bond angle (deg)	1.3	1.3	1.4	1.4	1.3	1.3
average B-factor						
protein atoms (Å ²)	14.4	20.5	18.4	44.0	22.9	26.6
ligand atoms (Å ²)		13.6	11.6	32.6	16.1	18.6
waters (Å ²)	26.1	27.5	31.6	47.2	28.9	24.4
Ramachandran plot ^g						
most favored regions (%)	97.9	97.6	97.0	96.7	97.3	96.5
allowed regions (%)	2.1	2.4	3.0	3.2	2.7	3.5
outlier regions (%)	0.0	0.0	0.0	0.1	0.0	0.0
PDB ID entry	6AYM	6AYO	6AYQ	6AYR	6AYS	6AYT

^aValues in parentheses refer to the highest resolution shell.

^b $R_{\text{merge}} = (\sum_i h k l \sum_j |I_i(hkl) - \langle I(hkl) \rangle|) / (\sum_i h k l \sum_j \langle I_i(hkl) \rangle)$, where $I_i(hkl)$ is the intensity of the i th measurement of reflection (hkl) and $\langle I(hkl) \rangle$ is its mean intensity.

^c $R_{\text{rim}} = (\sum_i h k l (1/(N_{hkl} - 1))^{1/2} \sum_j |I_i(hkl) - \langle I(hkl) \rangle|) / (\sum_i h k l \sum_j \langle I_i(hkl) \rangle)$, where $I_i(hkl)$ is the intensity of the i th measurement of reflection (hkl) , $\langle I(hkl) \rangle$ is its mean intensity and N is the number of measurements.

^d I is the integrated intensity and $\sigma(I)$ is its estimated standard deviation.

^e $R_{\text{work}} = (\sum_i h k l |F_o - F_c|) / \sum_i h k l F_o$ where F_o and F_c are the observed and calculated structure factors.

^f R_{free} is calculated as for R_{work} but from a randomly selected subset of the data (5%), which were excluded from the refinement calculation.

^gCalculated by MOLPROBITY.