Tight binding enantiomers of pre-clinical drug candidates

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1. Introduction

Transition state analogues, potent inhibitors of the target enzyme.²⁻⁴ Both the enzyme, and more often than not, the inhibitors are chiral. With regards to α -amino acids and sugars this chirality is often manifested in the form of L- and D-modifications, respectively. Surprisingly, given the chirality of the enzyme target, and biological targets in general, the D form of the α -amino acids and the L-form of the sugars often retain or offer improved biological activity when compared with their enantiomers.⁵⁻¹⁰ Coupled with this improved biological activity, enantiomers may exhibit useful pharmacological activities in terms of improved solubility, bioavailability, stability, and reduced toxicity in vivo as well as extending the patent life of existing drugs.¹¹ In particular the study of the biological activity of a variety of L-nucleosides has often revealed surprising activity^{8,9} and our laboratories have demonstrated the biological activity of the L-nucleoside analogues^{12,13} of the immucillins.¹⁴

Immunocillins, based on transition state analogue design of purine nucleoside phosphorylase (PNP), have been described.¹⁴ Syntheses of the enantiomers of the two clinical leads, Forodesine¹⁵⁻¹⁸ and Ulodesine,¹⁹⁻²¹ have been reported together with their biological activity (Fig. 1). (3R,4S) – MT-DADMe-ImmH (MTDIH, *ent*-1) is a related compound and its activity as a PNP inhibitor has also been described.²² Recently the activity of MTDIH against the newly described enzyme methylthioinosine phosphorylase (MTIP) has also been published and the potential of inhibitors of MTIP as antibiotics discussed.²³

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ABSTRACT

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MTDIA is a picomolar transition state analogue inhibitor of human methylthioadenosine phosphorylase and a femtomolar inhibitor of *E. coli* methylthioadenosine nucleosidase. MTDIA has proven to be a non-toxic, orally available pre-clinical drug candidate with remarkable anti-tumour activity against a variety of human cancers in mouse xenografts. The structurally similar compound MTDIH is a potent inhibitor of human and malarial purine nucleoside phosphorylase (PNP) as well as the newly discovered enzyme, methylthioinosine phosphorylase, isolated from *Pseudomonas aeruginosa*. Since the enantiomers of some pharmaceuticals have revealed surprising biological activities, the enantiomers of MTDIH and MTDIA, compounds **1** and **2** respectively, were prepared and their enzyme binding properties studied. Despite binding less tightly to their target enzymes than their enantiomers compounds **1** and **2** are nanomolar inhibitors.

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-DADMe-ImmA (MTDIA, *ent*-2)²⁴ (Fig 1) and hence we are interested in the synthesis of its enantiomer. In particular, where whole-body methylthioadenosine phosphorylase (MTAP) is blocked by MTDIA,²⁴ dramatic effects are observed in mouse xenografts of a variety of human tumours.^{25,26} Also, MTDIA is a

Figure 1

Therefore, given the biological activity of MTDIH and MTDIA, and the surprising activity of the enantiomers of Forodesine and Ulodesine, we investigated the synthesis and enzyme inhibitory activity of their enantiomers, (3*S*,4*R*)-MTDIH **1** and (3*S*,4*R*)-MTDIA **2** (Fig. 2). In the case of **2** we also investigated its binding to *Ec*MTAN both in terms of the thermodynamics of binding and through the crystal structure of *Ec*MTAN with **2** in the active site.

2. Results and Discussion

2.1. Chemistry

Previously, Clinch *et al* described the large scale synthesis of (3*R*,4*R*)-hydroxymethylpyrrolidin-3-ol from diethyl maleate *via* isoxazolidine **3** (Scheme 1).³⁰ The key step in the synthesis involves an enantioselective enzymatic resolution of compound **4**, using the lipase Novozyme 435, to afford the carboxylic acid (3*S*,4*R*)-**5** and the ethyl ester (3*R*,4*S*)-**6**. Compound (3*S*,4*R*)-**5** has been converted to *ent*-**7** and then to (3*R*,4*R*)-hydroxymethylpyrrolidin-3-ol *ent*-**9**. The other product from the enantioselective resolution process, compound (3*R*,4*S*)-**6** can be used to synthesise the desired enantiomers of MTDIA and MTDIH. Therefore reduction of **6** was achieved with borane, generated *in situ* from BF₃·OEt₂ and NaBH₄, to afford diol **7** in good yield which could be readily separated from a minor contaminant, the ethyl ether **8** (8%). Hydrogenolysis of the *N*-benzyl protecting group was readily achieved using Pearlman's catalyst where the reaction solvent, methanol, was doped with 1% aqueous ammonia (v:v). Boc protection of amine **9** could be

carbamate **10** in excellent overall yield for the two steps. Methanesulfonylation of compound **10** was carried out at -60 °C and although the reaction was not entirely regioselective, the over-reacted side-product could be carried through to the next step along with the desired product **11**, and the resulting impurity removed later by chromatography. Dibutyltin oxide can also be used to achieve regioselective mesylation, however due to the inherent toxicity of organotin compounds we were not prepared to use this method. Treatment of the crude product from the mesylation step with sodium thiomethoxide at room temperature afforded the Boc protected 5-methylthiopyrrolidine **12** in moderate yield for the two steps. Removal of the Boc protecting group was achieved using concentrated HCl in methanol to afford the desired amine hydrochloride **13**, the right-hand side of the two target molecules, in quantitative yield.

Synthesis of the 9-deazapurine component of the target molecule **1**, 9-deazahypoxanthine, has been well described and it was prepared using a method reported previously.^{31,32}

The synthesis of 9-deazaadenine is less well described³³⁻³⁵ and although our preferred route has been previously outlined³⁶ the synthetic details of this robust and scalable process have not been previously revealed (Scheme 2).

Ethyl(ethoxymethylene)cyno acetate (**14**) was treated with aminooacetonitrile to afford enamine **15** in excellent yield. The enamine nitrogen of compound **15** was protected using methylchloroformate, again in excellent yield, to afford **16** and this was then cyclised using DBU to provide pyrrole **17** in good yield. Reaction of pyrrole **17** with formamidine acetate in ethanol gave ester **18** which was efficiently decarboxylated to afford 9-DAA as a crystalline solid in 25% overall yield for the 5 steps without chromatography.

Target compounds **1** and **2** were synthesized in a similar fashion to that previously described for their enantiomers²⁴ utilizing Mannich chemistry.³⁷ The amine hydrochloride **13** was dissolved in a 1,4-dioxane:water mixture which was buffered with sodium acetate. Formaldehyde was added followed by either 9-deazahypoxanthine or 9-deazaadenine and the resulting mixtures heated to 95 °C to afford the target compounds **1** and **2**.

Scheme 1. (a) Novozyme 435, H₂O, acetone, pH 7.5, 27 °C, reflux; (b) BF₃·OEt₂, NaBH₄, THF, 0 °C → room temperature; (c) Pd(OH)₂, H₂, conc NH₄OH, MeOH, room temperature; (d) Boc₂O, MeOH, room temperature; (e) MsCl, Hunig's base, CH₂Cl₂, -60 °C; (f) NaSMc, DMF, room temperature; (g) MeOH; conc HCl, room temperature.

Scheme 2. (a) Aminoacetonitrile bisulfate, NaOAc, MeOH, room temperature; (b) Methyl chloroformate, Et₃N, CH₂Cl₂, room temperature; (c) CH₂Cl₂, DBU, MeOH, room temperature; (d) Formamidine acetate, EtOH, reflux; (e) KOH, H₂O, reflux.

Scheme 3. (a) aq Formaldehyde, NaOAc, 1,4-dioxane, H₂O, 95 °C

in good to moderate yields, respectively (Scheme 3).

2.2. Inhibition Studies

The inhibition of human and *Plasmodium falciparum* PNP, *Pseudomonas aeruginosa* MTIP, human MTAP and *Escherichia coli* MTAN was evaluated with enantiopure immucillins **1**, *ent*-**1**, **2** and *ent*-**2** (Table 1). While the inhibition constants of compounds **1** and **2** indicate binding that is considerably weaker for their respective target enzymes, than their enantiomers, somewhat surprisingly, they remain high to low-range nanomolar inhibitors for all enzymes.

Table 1. Summary of K_i values (nM).

1		Ent-1	
Enzyme	K_i	K_i^*	K_i
<i>Hs</i> PNP	110 ± 14	84 ± 17	0.3 ± 0.01
<i>Pf</i> PNP	298 ± 37	93 ± 4	11 ± 4
<i>Pa</i> MTIP	8.6 ± 0.2		0.8 ± 0.1
2		Ent-2	
Enzyme	K_i	K_i^*	K_i
<i>Hs</i> MTAP	750 ± 10		0.53 ± 0.05
<i>Ec</i> MTAN	4.5 ± 0.5		0.048 ± 0.003

The L-enantiomer compound **1** bound to human PNP some 1200-fold less tightly to the enzyme than (3*R*,4*S*)-MTDIIH (*ent*-**1**). Despite this difference, **1** remains a nanomolar inhibitor of human PNP and in some drug design programs, could be considered a potent inhibitor. Against MTIP, compound **1** was a low nanomolar inhibitor with slow-onset tight binding characteristics and was only 2-3 times less potent than its enantiomer. Alternatively, compound **2** exhibited no slow-onset tight binding inhibition against either MTAP or MTAN and showed some 3-4 orders of magnitude weaker binding to these enzymes than its enantiomer *ent*-**2**.

2.3. Thermodynamics of binding for (3*S*,4*R*)-MTDIA **2**

The energetics of binding of compound **2** to *E. coli* MTAN is different from that of *E. coli* MTAN with *ent*-**2**. With *ent*-**2**, binding was driven by large enthalpic values: -15.2 kcal mol⁻¹ at the first active site; and -13.5 kcal mol⁻¹ at the second active site of the MTAN homodimer.³⁸ These large enthalpy contributions were accompanied by smaller entropic binding contributions of -2.5 kcal mol⁻¹ at the first active site and 3.4 kcal mol⁻¹ at the second active site.³⁸ When **2** binds, the enthalpic binding contribution is reduced to -8.1 and -5.6 kcal mol⁻¹ at the first and second sites, respectively. The entropic binding contribution is smaller but favorable at -3.6 and -4.4 kcal mol⁻¹ at the first and second binding sites generated in the case of **2** is likely due to the release of bound Overall, the thermodynamic signature suggests different modes of binding between the enantiomers of MTDIA and *E. coli* MTAN, consistent with the crystal structures.

2.4. Crystal structure of EcMTAN·(3*S*,4*R*)-MTDIA **2**.

Consistent with all MTAN structures deposited thus far with DADMe-Immucillin-based inhibitors bound, the purine-like portion of **2** is hydrogen bonded to Ile152 and Asp197. Likewise, the 5'-alkylthio group in both enantiomers have similar conformations, and do not make any strong interactions. In contrast, the aza-sugar portion of the molecule must adopt a very different orientation in **2** compared to *ent*-**2** (Figure 3). This is illustrated by the torsion angle C9-C10-N1'-C1', which changes almost 110° from 46.6° to -60.3°. The aza-sugar in both enantiomers (in the bound state) is in an N1'-endo conformation. The latter inhibitor *ent*-**2** is a transition state analogue, which matches the stereochemistry of the adenosine-derived substrates, whereas the former inhibitor **2** has the opposite stereochemistry at C3' and C4'. The hydrogen bonding contacts to the aza-sugar are somewhat different between the enantiomers. Thus far, all deposited structures with the (3*R*,4*S*)-alkylthio-DADMe-Immucillin A based inhibitors have a water molecule (and sometimes two) distal to the aza-sugar in the active site cavity: the "nucleophilic" water, which would act to hydrolyze the native substrate(s); and a supporting water molecule if space permits. Neither of these water molecules are present in the **2**-bound EcMTAN structure. In the (3*R*,4*S*)-inhibitor-bound structures, N1' is always hydrogen bonded to the nucleophilic water, which in turn is hydrogen bonded to both Glu12 and Glu174.

Additionally, the C3'-hydroxyl group appears to make a single hydrogen bond to Glu174. In contrast, in the (3*S*,4*R*)-inhibitor-bound structure N1' faces away from Glu12 and instead, hydrogen bonds to the neighboring Ser76. The C3'-hydroxyl group now has a bifurcated hydrogen bond to Glu174. The capacity of EcMTAN to bind **2** appears to be positively influenced by Ser76, which provides a key hydrogen bonding interaction. In MTANs from several other organisms, such as *C. difficile*, *M. tuberculosis* and *H. pylori*, the analogous residue is a valine and so the binding affinity to **2** might be expected to be much worse. Comparison of the *ent*-**2** and **2**-bound EcMTAN structures shows the active site residues are positioned very similarly (Figure 3), with residues closer than 5 Å to the inhibitors having an RMSD of 0.70 Å (167 atoms in alignment). As expected, the alignment for the entire structure is also very similar, having an RMSD of 0.91 Å (3344 atoms in alignment). In both alignments, no atoms were excluded due to poor fit.

3. Conclusion

Enantiomers of two compounds that show promise as drug candidates have been prepared and assayed against a series of target enzymes. While not as potent as their enantiomers, **1** and **2** are still sub-nanomolar inhibitors and their biological activity may warrant further investigation. The thermodynamic data suggests different modes of binding by **2** and *ent*-**2** which are borne out by the structural data. A structural analysis of **2** bound to *E. coli* MTAN shows that in order to accommodate the opposite stereochemistry the water molecules normally found in the active site are absent and new hydrogen bond connection is revealed to Ser76.

4. Experimental protocols

4.1. Synthesis

Air sensitive reactions were performed under argon. Organic solutions were dried over anhydrous MgSO₄ and the solvents were evaporated under reduced pressure. Anhydrous and chromatography solvents were obtained commercially and used without any further purification. Thin layer chromatography (t.l.c.) was performed on glass or aluminium sheets coated with 60 F254 silica gel. Organic compounds were visualized under uv light or use of a dip of ammonium molybdate (5 wt %) and cerium(IV) sulfate 4 H₂O (0.2 wt %) in aq. H₂SO₄ (2 M), one of I₂ (0.2 %) and KI (7%) in H₂SO₄ (1 M), or 0.1 % ninhydrin in EtOH. Chromatography (flash column) was performed on silica gel (40-63 μm) or on an automated system with continuous gradient facility. Optical rotations were recorded at a path length of 10 cm and are in units of 10⁻¹deg cm² g⁻¹; concentrations are in g/100 ml. ¹H NMR spectra were measured in CDCl₃, CD₃OD, DMSO d₆ (internal Me₄Si, δ 0) or D₂O (HOD, δ 4.79), and ¹³C NMR spectra in CDCl₃ (centre line, δ 77.0), CD₃OD (centre line, δ 49.0), DMSO d₆ (centre line, δ 39.5) or D₂O (no internal reference or internal CH₃CN, δ 1.47 where stated). Assignments of ¹H and ¹³C resonances were based on 2D (¹H-¹H DQF-COSY, ¹H-¹³C HSQC, HMBC) and DEPT experiments. Positive electrospray mass spectra were recorded on a Q-TOF Tandem Mass Spectrometer. Microanalyses were performed by the Campbell Microanalytical Department, University of Otago, Dunedin, New Zealand.

(3*S*,4*S*)-1-Benzyl-4-(hydroxymethyl)pyrrolidin-3-ol (7). To a suspension of lactam **6** (9.90 g, 37.6 mmol) and sodium borohydride (5.69 g, 150 mmol) in dry THF (190 mL) was added boron trifluoride diethyl etherate (23.2 mL, 188 mmol) dropwise at 0 °C and the resulting reaction mixture was stirred for 3 d at rt. The reaction was then quenched carefully with methanol under ice cooling and the solvent was concentrated in vacuo. 6 M hydrochloric acid (60 mL) was added and after being stirred for 10 min the mixture was again concentrated in vacuo. Subsequently, a 15% aqueous sodium hydroxide solution was added (*ca.* 60 mL) to the resulting residue until the pH reached 14. After concentrating the mixture again the residue was suspended in chloroform, stirred for 1 h at room temperature and filtered through Celite®. The crude product was then purified by silica gel chromatography (eluent methanol/chloroform 1:9 → 1:4) to yield 4.10 g (53%) of the title compound **7** as a colorless oil. In addition, 726 mg (8%) of the corresponding ethyl ether **8** were isolated as a by-product.

(3*S*,4*S*)-1-Benzyl-4-(hydroxymethyl)pyrrolidin-3-ol (7): $[\alpha]_{\text{D}}^{22} = -33.1$ (*c* 1.095, MeOH); ^1H NMR (500 MHz, CD_3OD) δ 7.34-7.28 (m, 4H), 7.26-7.21 (m, 1H), 3.99 (dt, *J* = 4.1 Hz, 6.3 Hz, 1H); 3.64 (d, *J* = 12.7 Hz, 1H), 3.62 (dd, *J* = 5.9, 10.7 Hz, 1H), 3.55 (d, *J* = 12.7 Hz, 1H), 3.50 (dd, *J* = 7.7, 10.7 Hz, 1H), 2.89 (dd, *J* = 8.1, 9.5 Hz, 1H), 2.72 (dd, *J* = 6.3, 10.1 Hz, 1H), 2.56 (dd, *J* = 4.1, 10.1 Hz, 1H), 2.33 (dd, *J* = 6.6, 9.7 Hz, 1H), 2.21-2.13 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 139.36, 130.28, 129.36; 128.33, 74.17, 64.25, 63.10, 61.52, 51.19; HRMS (ESI) *m/z* calcd for $\text{C}_{12}\text{H}_{17}\text{NO}_2\text{H}^+$ 208.1332, obsd 208.1343.

(3*S*,4*S*)-1-Benzyl-4-(ethoxymethyl)pyrrolidin-3-ol (8): Yellow oil; $[\alpha]_{\text{D}}^{20} = -24$ (*c* = 1.21, MeOH). ^1H NMR (500 MHz,

ammonia (0.2 mL, 28%, 13 mmol, and methanol (20 mL) were stirred under an atmosphere of hydrogen gas (0.026 g, 13.03 mmol) for 14 h. The resulting mixture was filtered through Celite® and concentrate in vacuo to afford a crude syrup. The crude product was purified by flash chromatography (eluent 1:1 1,4-dioxane:conc NH_4OH) to afford (3*S*,4*S*)-3-hydroxy-4-hydroxymethylpyrrolidine (**9**) (1.34 g, 88%). The ^1H and ^{13}C NMR were in agreement with the data for the (3*R*,4*R*)-enantiomer reported by Filichev et al.³⁹ Boc anhydride (4.99 g, 23 mmol) was added to a solution of **10** (1.34 g, 11.4 mmol) in methanol (20 mL) and the resulting stirred for 1 h. The solution was concentrated in vacuo and the crude residue purified by chromatography (eluent 10 → 20% MeOH: CHCl_3) to afford **10** (2.20 g, 89 % yield) as a colourless syrup. The ^1H and ^{13}C NMR were in agreement with the data for the (3*R*,4*R*)-enantiomer reported by Evans et al.⁴⁰ $[\alpha]_{\text{D}}^{20} = -15.2$ (*c* = 1, MeOH) equal and opposite to that reported for the (3*R*,4*R*)-enantiomer.⁴⁰

(3*S*,4*R*)- 3-Hydroxy-4-(methylthiomethyl)pyrrolidine (13). Hunig's base (3.53 mL, 20.25 mmol) was added to a solution of (3*S*,4*S*)-**10** (2.2 g, 10.13 mmol) in dichloromethane (35 mL), and the resulting mixture cooled to -60 °C. Methanesulfonyl chloride (0.784 mL, 10.1 mmol) was added dropwise and the internal temperature maintained at -60 °C. After 30 minutes the reaction was judged to be complete and was diluted with chloroform and washed with water, 10% HCl and satd NaHCO_3 , dried and concentrated in vacuo. The crude mesylate **11** (2.99 g), was committed to the next synthetic step without purification or characterisation.

Sodium thiomethoxide (1.42 g, 20.2 mmol) was added to a solution of mesylate **11** (2.99 g, 10.1 mmol) in dimethylformamide (40 mL) and the resulting suspension left to stir for 14 h.

The mixture was then diluted with toluene and washed with water, brine, dried (MgSO_4), and concentrated in vacuo.

The crude residue was purified by chromatography (eluent 20% → 60% → 100% EA) to afford compound **12**, presumably (3*S*,4*R*)-1-*tert*-butoxycarbonyl-3-hydroxy-4-(methylthiomethyl)pyrrolidine (0.96 g) as a syrup which was committed to the next step without characterisation. Concentrated HCl (2 mL, xs) was added to a solution of compound **12** (0.96 g, 3.9 mmol in methanol (2 mL) and the solution concentrated in vacuo. The resulting residue was dissolved in additional conc HCl (2 mL) and concentrated in vacuo to afford the title compound **13** (0.71 g, 38 % yield for 3 steps) as a low melting crystalline solid. ^1H NMR (500MHz, D_2O) δ 4.41 (brs, 1H), 3.68 (dd, *J* = 12, 6.5 Hz, 1H), 3.51 (dd, *J* = 12.6, 5.1 Hz, 1H), 3.29 (brd, *J* = 12.6 Hz, 1H), 3.22 (dd, *J* = 12.3, 5.1 Hz, 1H), 2.71 (q, *J*

Figure 3. EcMTAN bound to (3*S*,4*R*)-MTDIA (left) and (3*R*,4*S*)-MTDIA (right). The key residues are shown as sticks. The nearby residues are shown as lines.

CD_3OD) δ 7.34-7.28 (m, 4H), 7.26-7.22 (m, 1H), 3.98 (dt, *J* = 4.1 Hz, 6.3 Hz, 1H); 3.63 (d, *J* = 12.7 Hz, 1H), 3.54 (d, *J* = 12.6 Hz, 1H), 3.51-3.46 (m, 3H), 3.38 (dd, *J* = 7.7, 9.3 Hz, 1H), 2.88 (dd, *J* = 7.9, 9.2 Hz, 1H), 2.88 (dd, *J* = 7.9, 9.2 Hz, 1H), 2.70 (dd, *J* = 6.3, 10.1 Hz, 1H), 2.29 (dd, *J* = 6.7, 9.4 Hz, 1H), 2.27-2.20 (m, 1H), 1.16 (t, *J* = 7 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 139.38, 130.29, 129.35, 128.33, 74.37, 72.86, 67.48, 62.94, 61.51, 57.56; HRMS (ESI) *m/z* calcd for $\text{C}_{14}\text{H}_{21}\text{NO}_2\text{H}^+$ 236.1645, obsd 236.1644.

(3*S*,4*S*)-*N*-*tert*-Butoxycarbonyl-3-hydroxy-4-hydroxymethylpyrrolidine (10). A suspension of (3*S*,4*S*)-*N*-benzyl-3-hydroxy-4-hydroxymethylpyrrolidine (**7**) (2.7 g, 13.03 mmol, Pearlman's catalyst (200 mg, 1.879 mmol, aqueous

= 16.9, 10.5 Hz, 1H), 2.55 (m, 2H), 2.14 (s, 3H). ¹³C NMR (500MHz, D₂O) δ 73.5, 51.5, 48.6, 45.2, 34.3, 14.9. HRMS (ESI) m/z calcd for C₆H₁₃NOSH⁺ 148.0796, obsd 148.0803. [α]_D²⁰ = -34.5 (c = 1, MeOH) equal and opposite to that of the (3*R*,4*S*)-enantiomer.⁴¹

(E)-Ethyl 2-cyano-3-(cyanomethylamino)acrylate (15). A suspension of aminoacetonitrile bisulfate (**14**) (73 g, 474 mmol) and sodium acetate (117 g, 1421 mmol) in methanol was stirred at r.t. for 20 min. Ethyl(ethoxymethylene) cyano acetate (80 g, 474 mmol) was then added in one portion with vigorous stirring and the mixture left for 1 h. The mixture was evaporated to dryness and the solid suspended in a satd. aq. NaHCO₃ solution (1.0 L) which was then extracted with EtOAc (2 x 400 mL). The organic layers were combined and washed with water (250 mL), dried (MgSO₄), filtered and the solvent evaporated in vacuo to afford compound **15** (83.4 g, 98 % yield) as a yellow solid (characterised as a 60:40 isomeric mixture, H' = minor isomer protons). M.p. 95 °C. ¹H NMR (500 MHz, d₆-DMSO) δ 9.27 (brs, 1H), 9.00 (brs, 1H), 8.16 (s, 1H), 7.86 (brd, *J* = 11.3 Hz, 1H), 4.53 (s, 2H), 4.44 (s, 2H), 4.17 (q, *J* = 7.2 Hz, 2H), 4.14 (q, *J* = 7.1 Hz, 2H), 1.23 (t, *J* = 7.2 Hz, 3H), 1.21 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (125 MHz, d₆-DMSO) δ 165.7, 164.3, 160.1, 159.9, 118.2, 116.9, 116.9, 115.7, 72.7, 71.9, 60.1, 36.8, 36.1, 14.3, 14.0. HRMS (ESI) m/z calcd for C₈H₉N₃O₂Na 202.0592, obsd 202.0590.

(E)-Ethyl 2-cyano-3-((cyanomethyl)(methoxycarbonyl)amino)acrylate (16). Compound **15** (76 g, 424 mmol) was suspended in CH₂Cl₂ (1.0 L) and to this mixture was added methyl chloroformate (45.9 ml, 594 mmol). Triethylamine (83 ml, 594 mmol) was then added dropwise at such a rate as to ensure the reaction temperature is maintained below 30 °C. After the addition was complete the mixture was stirred for a further 30 minutes at which point the reaction was complete. The organic layer was then washed with water (500 ml) and satd. aq. NaHCO₃ ensuring the pH of the aqueous layer is basic. The organic layer was dried (MgSO₄), filtered and concentrated in vacuo to afford **16** (96 g, 95%) as a dark red syrup. ¹H NMR (500 MHz, d₆-DMSO) δ 8.44 (1H, s), 5.07 (2H, s), 4.27 (q, *J* = 7.1 Hz, 2H), 3.94 (3H, s), 1.27 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (125 MHz, d₆-DMSO) δ 162.6, 151.9, 149.4, 115.3, 114.0, 83.6, 62.1, 56.0, 34.2, 14.0. HRMS (ESI) m/z calcd for C₁₀H₁₁N₃O₄Na 260.0647, obsd 260.0652.

Ethyl 4-amino-5-cyano-1H-pyrrole-3-carboxylate (17). Compound **16** (109 g, 460 mmol) was dissolved in CH₂Cl₂ (1.0 L, 460 mmol) and DBU (34.6 mL, 230 mmol) added dropwise. A small exotherm occurred (19 → 28 °C) on addition and the reaction was complete after 30 min. 10% aq. HCl (250 mL) was added to the organic layer and the two layers stirred together for 10 minutes. The two layers were partitioned and the organic layer washed with an additional quantity of 10% aq. HCl (250 ml), satd. aq. NaHCO₃, and water. The organic layer (MgSO₄) was dried, filtered and concentrated in vacuo to afford **17** (107 g, 98%) as a brown solid (compound characterised in ~90% purity). M.p. 205 °C. ¹H NMR (500 MHz, d₆-DMSO) δ 11.8 (brs, 1H), 7.35 (s, 1H), 5.65 (s, 2H), 4.20 (q, *J* = 7.1 Hz, 2H), 1.26 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (125 MHz, d₆-DMSO) δ 164.0, 146.0, 127.4, 114.8, 102.3, 84.4, 59.1, 14.3. HRMS (ESI) m/z calcd for C₈H₉N₃O₂Na 202.0592, obsd 202.0591.

Ethyl 4-amino-5H-pyrrolo[3,2-*d*]pyrimidine-7-carboxylate (18). A mixture of compound **17** (107 g, 451 mmol) and formamidine acetate (70.4 g, 677 mmol) in ethanol (1.0 L, 451 mmol) was heated at reflux overnight. The mixture was cooled to 0 °C and filtered, washing the solid with cold ethanol (1.0 L) to afford ethyl 4-amino-5-cyano-1H-pyrrole-3-carboxylate (42.4

g, 206 mmol, 45.6 % yield) as a gun-metal grey solid. M.p. >300 °C. ¹H NMR (500MHz, d₆-DMSO) δ 8.22 (s, 1H), 8.17(s, 1H), 4.25 (q, *J* = 7.0 Hz, 2H), 1.30 (t, *J* = 7.0 Hz, 3H).³⁵ ¹³C NMR (125 MHz, d₆-DMSO) δ 163.0, 152.0, 150.9, 145.0, 134.0, 114.5, 107.0, 58.9, 14.5.

9-Deazaadenine.³⁵ Potassium hydroxide (34.1 g, 517 mmol) was added to a suspension of ethyl 4-amino-5-cyano-1H-pyrrole-3-carboxylate (42.62 g, 207 mmol) in water (500 ml) and refluxed for 24 h. The mixture was cooled to r.t. concentrated in vacuo and resuspended in water (100 ml). The resulting suspension was filtered to afford a brown solid which was washed with water (50 ml) and ether (100 ml), and then air-dried to afford 9-deazaadenine (18 g, 65%) as a tan solid. M.p. >300 °C. ¹H NMR (500MHz, d₆-DMSO) δ 8.12 (s, 1H), 7.51 (brd, *J* = 2.1 Hz, 1H), 6.36 (brd, *J* = 2.1 Hz, 1H). ¹³C NMR (125 MHz, d₆-DMSO) δ 150.4, 150.3, 147.2, 127.8, 113.6, 101.2.

(3*S*,4*R*)-1-[(9-Deazahypoxanthin-9-yl)methyl]-3-hydroxy-4-(methylthiomethyl)pyrrolidine (1). Aqueous formaldehyde (0.245 ml, 1.6 mmol, 37% bw) was added dropwise to a mixture of amine hydrochloride **13** (200 mg, 1.1 mmol) and sodium acetate (179 mg, 2.2 mmol) in water (2 ml) and 1,4-dioxane (1 ml) mixture. After 5 minutes 9-deazahypoxanthine (0.177 g, 1.3 mmol) was added and the resulting suspension heated to 95 °C for 14 h. The crude reaction mixture was absorbed onto silica gel and the resulting residue purified by flash chromatography on silica gel (15% 7*N* NH₃ in MeOH:CHCl₃ → 20% → 30%) to afford the title compound **1** (220 mg, 69 % yield) as a white solid. M.p. 239-240 °C. ¹H NMR (500 MHz, d₆-DMSO) δ 7.79 (s, 1H), 7.28 (s, 1H), 4.84 (brs, 1H), 3.79 (bs, 1H), 3.62 (dd, *J* = 23.4, 13.3 Hz, 2H), 2.78 (dd, *J* = 9.2, 7.8 Hz, 1H), 2.72 (dd, *J* = 9.6, 6.6 Hz, 1H), 2.62 (dd, *J* = 12.7, 5.9 Hz, 1H), 2.40 (m, 2H), 2.24 (dd, *J* = 9.3, 6.2 Hz, 1H), 2.02 (s, 3H). ¹³C NMR (125 MHz, d₆-DMSO) δ 153.7, 143.4, 141.2, 126.9, 117.5, 113.3, 74.9, 61.5, 57.3, 47.7, 46.7, 36.8, 14.9. HRMS (ESI) m/z calcd for C₁₃H₁₈N₄O₂SH⁺ 295.1229, obsd 295.1226. [α]_D²⁰ = -45.9 (c = 1, MeOH). equal and opposite to that of the (3*R*,4*S*)-enantiomer.⁴² Found: C, 53.08; H, 6.30; N, 19.08; S, 10.79. C₁₃H₁₈N₄O₂S requires: C, 53.04; H, 6.16; N, 19.03; S, 10.89.

(3*S*,4*R*)-1-[(9-Deazaadenin-9-yl)methyl]-3-hydroxy-4-(methylthiomethyl)pyrrolidine (2). Aqueous formaldehyde (0.257 ml, 1.72 mmol, 37% bw) was added to a mixture of amine hydrochloride **13** (210 mg, 1.14 mmol) and sodium acetate (188 mg, 2.29 mmol) in water (2 ml) and 1,4-dioxane (1 ml). After 5 minutes 9-deazaadenine (0.184 g, 1.372 mmol) was added and the resulting suspension warmed to 95 °C for 3 h. The crude reaction mixture was absorbed onto silica gel and the resulting residue purified by flash chromatography on silica gel (15% 7*N* NH₃ in MeOH:CHCl₃ → 20% → 30%) to afford the title compound **2** (160 mg, 48%) as a white solid. M.p. decomposed >180 °C. ¹H NMR (500 MHz, d₄-MeOH) 8.15 (s, 1H), 7.49 (s, 1H), 3.96 (dt, *J* = 6.3, 4.1 Hz, 1H), 3.83 (dd, *J* = 13.4, 9.8 Hz, 2H), 3.05 (dd, *J* = 9.7, 7.9 Hz, 1H), 2.85 (dd, *J* = 10.2, 6.4 Hz, 1H), 2.70 – 2.65 (m, 2H), 2.47 (dd, *J* = 12.7, 8.9 Hz, 1H), 2.38 (dd, *J* = 9.8, 7.1 Hz, 1H), 2.21 (m, 1H), 2.06 (s, 3H). ¹³C NMR (125 MHz, d₄-MeOH) 152.1, 151.0, 147.0, 130.1, 115.2, 112.5, 76.8, 62.3, 58.9, 48.9, 48.2, 38.1, 15.6. [α]_D²⁰ = -41.6 (c = 1.09, MeOH) equal and opposite to that of the (3*R*,4*S*)-enantiomer.⁴³ HRMS (ESI) m/z calcd for C₁₃H₁₉N₅OSH⁺ 294.1389, obsd 294.1385. Found: C, 51.91; H, 6.73; N, 23.04; S, 10.10. C₁₃H₁₉N₅OS.½H₂O requires: C, 51.63; H, 6.67; N, 23.16; S, 10.60.

4.2. Biology

Protein Preparation. The recombinant human and *P. falciparum* PNP's, *P. aeruginosa* MTIP and human MTAP were expressed and purified as previously described.²³ The recombinant *E. coli* MTAN was expressed in BL-21 DE3 Star (Invitrogen), IPTG inducible *E. coli* cells and purified using Ni-NTA chromatography. All five enzymes contain N-terminal His₆-tags, but are catalytic equivalents of the native proteins.

Inhibition Assays. Enzyme inhibition assays were carried out using xanthine oxidase as the coupling enzyme and monitored for 60-120 minutes. For the PNP's and MTIP, hypoxanthine formed by the phosphorolysis of inosine and MTI respectively, is converted to uric acid by xanthine oxidase (Sigma) and monitored spectrometrically at 293 nm ($\epsilon_{293} = 12.9 \text{ mM}^{-1}\text{cm}^{-1}$).⁴⁴ For the PNP's, the assay was initiated by the addition of either 0.5 nM (human) and 10 nM (*P. falciparum*) enzyme into a 1 mL reaction mixture containing 1 mM inosine, 50 mM potassium phosphate (pH = 7.5), with xanthine oxidase added to a final concentration of 60 milliunits. For *P. aeruginosa* MTIP, the assay was initiated by the addition of 1 nM enzyme into a 1 mL reaction mixture containing 2 mM MTI, 100 mM HEPES (pH = 7.4), 100 mM potassium phosphate, 5 mM dithiothreitol and 0.05 units of xanthine oxidase.

For both MTAP and MTAN reactions, the adenine formed by phosphorolysis of MTA, is converted to 2,8-hydroxyladenine by xanthine oxidase and monitored spectrophotometrically at 305 nm ($\epsilon_{305} = 15.5 \text{ mM}^{-1}\text{cm}^{-1}$).⁴⁵ For human MTAP, the reaction was initiated by the addition of 0.8 nM enzyme into a 1 mL reaction mixture containing 1 mM methylthioadenosine (MTA), 50 mM HEPES (pH = 7.4), 100 mM potassium phosphate (pH = 7.4), 1 mM dithiothreitol and 1 unit of xanthine oxidase. For *E. coli* MTAN, the reaction was initiated by the addition of 1 nM enzyme into a 1 mL reaction mixture containing 1 mM MTA, 100 mM HEPES (pH 7.8) and 1 unit of xanthine oxidase. Controls without inhibitor and without enzyme were included in all experiments. K_i values were obtained using the follow equation for competitive inhibition:

$$\frac{v'_0}{v_0} = \frac{K_m + [S]}{K_m + [S] + \frac{K_m[I]}{K_i}}$$

Where v'_0 and v_0 are initial rates in the presence and absence of inhibitor, respectively; K_m and $[S]$ are the Michaelis constant and concentration of either inosine (for both PNP's), MTI (for MTIP) and MTA (for MTAP and MTAN), respectively, and K_i and $[I]$ are the inhibition constant and inhibitor concentration, respectively. If $[I]$ is less than 10 times the enzyme concentration, the following correction is applied:

$$I' = I - \left(1 - \frac{v'_0}{v_0}\right) E_t$$

Where I' is the effective inhibitor concentration to be used in the equation for competitive inhibition and E_t is the final enzyme concentration. In some instances, a second slower rate phase is observed in progress curves of tight binding inhibitors. These steady state rates are taken instead ($v's/v_s$) and used in the inhibition equation above to determine the equilibrium inhibition constant K_i^* , which reflects the slow onset inhibition of the enzyme-inhibitor complex.

ITC Experimental details. Titrations were performed at 25°C in a VP-ITC (MicroCal). EcMTAN was dialyzed against charcoal overnight to remove any bound adenine. Further dialysis followed in 100 mM phosphate buffer, pH 7.8. The dialysate was filtered and used to wash the cell and syringe, and to dissolve the

ligand. Using 8 μL injections, the ligand, (3S, 4R)-MTDIA (225 μM) was titrated into the cell containing EcMTAN (25 μM of the single subunit) to post-saturation. Past saturation, only heats of dilution were observed. The data was fit in Origin7 (MicroCal) using a standard equation for the fitting of two independent binding sites. K_a was constrained to the externally determined K_i reported within.

Co-crystallization of *E. coli* MTAN with (3S,4R)-MTDIA. EcMTAN was expressed and purified as described previously.³⁸ The enzyme, with C-terminal TEV-cleavage site and HIS₆ tag intact, was concentrated to 10 mg/ml and incubated with five equivalents of inhibitor. The crystallization experiments was performed at 22 °C using the sitting drop vapor diffusion technique. Rod-shaped crystals (25 $\times$ 35 $\times$ 230 μm) of inhibitor-bound EcMTAN were obtained over 4 days using 0.1 M BIS-TRIS pH 7.0 and 2.4 M sodium malonate.

Data Collection and Processing. The inhibitor-bound *E. coli* nucleosidase crystal was flash cooled in liquid nitrogen, without auxiliary cryoprotectant. Diffraction data for this crystal was collected with 0.9793 Å wavelength radiation at the LRL-CAT beamline (Argonne National Laboratory) on a Rayonix 225 HE CCD detector to 1.75 Å resolution. Diffraction intensities were integrated and scaled with XDS.⁴⁶ The diffraction data statistics are summarized in Table 2.

Structure Determination. The structure was determined by molecular replacement in Molrep,⁴⁷ using the previously published structure of EcMTAN:(3R,4S)-MTDIA (PDB code 1Y6Q) as a search model. The refinement of the initial solution and subsequent refinements were carried out using a restrained refinement in Refmac,⁴⁸ using all data between 25.0 and 1.75 Å. Manual model rebuilding was carried out using Coot.⁴⁹ Weighted difference Fourier maps revealed ordered water molecules and strong unambiguous positive $F_o - F_c$ difference electron density corresponding to the inhibitor. The inhibitor model was generated using JLigand.⁵⁰ Water molecules with proper hydrogen-bonding coordination and electron densities greater than 1 RMSD and 3 RSMD in maps calculated with $2F_{\text{obs}} - F_{\text{calc}}$ and $F_{\text{obs}} - F_{\text{calc}}$ coefficients, respectively, were included in the model. All but two of the Ec-associated residues (residues 1-230) were sufficiently ordered enough to be built into the model, however none of the residues in the cleavage/HIS₆-tag could be modeled. The structure was refined to R_{cryst} 17.3% and R_{free} 19.3%. Analysis of the structures in Coot revealed good stereochemistry with one residue (SER155) falling into the disallowed regions of the Ramachandran plot. The refinement statistics are reported in Table 2.

Table 2. Data collection and refinement statistics for EcMTAN:(3S,4R)-MTDIA.

Data collection	
Space group	C222 ₁
No. of mol. in asym. unit	1
Cell dimensions	
a, b, c (Å)	72.010, 91.840, 69.530
Resolution (Å) ^a	50.0-1.75 (1.85-1.75)
No. of unique reflections ^a	38054 (7212)

R_{merge}^a	0.074 (0.629)
$I / \sigma I^a$	14.0 (2.9)
Completeness (%) ^{a,b}	84.1 (98.9)
Refinement	
Resolution (Å)	25.0-1.75
R_{cryst}	0.173
R_{free}	0.193
R.m.s deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.29
No. of atoms (average B-factor)	
Protein	1699 (23.5)
Inhibitor	20 (24.4)
Waters	132 (31.7)
Other	5 (29.5)
PDB entry	4YML

^aNumbers in parentheses indicate values for the highest resolution shell.

^bSeveral diffraction zones were excluded due to ice rings, resulting in a low total completeness.

Acknowledgments

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