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8-Mercaptoguanine derivatives as inhibitors of dihydropteroate synthase

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Abstract: Dihydropteroate synthase (DHPS) is an enzyme of the folate biosynthesis pathway which catalyzes the formation of 7,8-dihydropteroate (DHPT) from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) and *para*-aminobenzoic acid (pABA). DHPS is the long-standing target of the sulfonamide class of antibiotics that compete with pABA. In the wake of sulfa drug resistance, targeting the structurally rigid (and more conserved) pterin site has been proposed as an alternate strategy to inhibit DHPS in wild-type and sulfa drug resistant strains. Following work developing pterin-site inhibitors of the adjacent enzyme 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK), we now present derivatives of 8-mercaptoguanine, a fragment that binds weakly within both enzymes, and quantify sub- μ M binding using Surface Plasmon Resonance (SPR) to *Escherichia coli* DHPS (EcDHPS). Eleven ligand-bound EcDHPS crystal structures delineate the structure-activity relationship observed providing a structural framework for the rational development of novel, substrate envelope-compliant DHPS inhibitors.

Introduction

The sulfonamide class of antibiotics was discovered in the 1930s and, along with the related sulfone class, is still widely used today to treat infectious diseases including malaria, tuberculosis, toxoplasmosis, *Pneumocystis jirovecii* pneumonia (the most common fungal infection in HIV-infected patients), and methicillin-resistant *Staphylococcus aureus*.^[1] Sulfonamides target the folate-pathway enzyme dihydropteroate synthase (DHPS), which catalyzes the condensation of *p*-aminobenzoic acid (pABA) and 6-hydroxymethyl-7,8-dihydropterin-

pyrophosphate (DHPPP) into dihydropteroate (DHPT) (Figure 1). Sulfonamides exert their effect through competing with pABA by reacting to form “dead-end” drug-pterin adducts, depleting cellular DHPPP as well as inhibiting downstream enzymes.^[2]

Figure 1. (A) Reaction pathway of DHPS showing the formation of DHPT from DHPPP and pABA. (B) Chemical structures of DHPS inhibitors. (C) Structure of the EcDHPS/ptericoic acid complex displayed. The ptericoic acid molecules are displayed in gray.

Despite decades of success in the clinic, mechanistic data is still emerging to aid the rational design of DHPS inhibitors. Crystallization studies have unraveled the catalytic mechanism and the structural basis behind sulfa drug resistance.^[3] Structural data has shed light on how the off-target inhibition of sepiapterin reductase has led to the neurological side effects of sulfa drugs following treatment.^[4] These structural details and other studies promise to help the development of newer generation sulfa drugs with improved therapeutic effect and longevity.^[5] Sulfa drug resistance rapidly emerged after usage and is predominantly a consequence of point mutations in the folP gene altering residues near to the pABA site.^[6]

Given the dearth of new antibiotic classes, the pterin-binding site of DHPS has been recognized as an alternative druggable pocket to reinvigorate the design of DHPS inhibitors. Crystal structures of DHPS from various organisms^[3, 7] reveal the pterin pocket to be rigid and lined with highly conserved residues located deep in the enzyme, in stark contrast to the pABA site which is nestled in the flexible loop regions. Taking advantage of these properties of the pterin pocket (which is

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required for substrate specificity) is potentially beneficial; under selective pressure, viable mutations conferring resistance to pterin-site inhibitors would be difficult to evolve without compromising activity. The conserved nature of the pterin pocket indicates that a suitably designed pterin-site agent could potentially elicit broad-spectrum activity and be effective against organisms that have become sulfa drug resistant.^[8] There are however potential difficulties targeting the substrate site which derive from drugging a pocket that is highly selective for a rather polar substrate that binds with high affinity.

The molecular details of the transition state have been trapped *in crystallo* revealing interesting details regarding the shape, loop conformational changes, and intermolecular interactions within the substrate binding envelope that can be used to guide affinity, selectivity, and monitor substrate-envelope 'compliance' during inhibitor design. Such compliance, according to the 'substrate-envelope hypothesis',^[9] would prevent intermolecular interactions beyond those available to the substrate and therefore limit mutations that are tolerated by the substrate but not by any inhibitors. A fragment approach to target allosteric pockets away from the active site may offer a novel way to perturb DHPS function^[10] and potentially mitigate difficulties associated with polar substrate-like compounds. ~~However, such pockets are less conserved across various species and would be more easily modified through mutations compared to the pocket around the substrate envelope.~~

Interest in pterin-site inhibitors started in the 1970s, when 4,5-dioxo-1,4,5,6-tetrahydropyrimido[4,5-c]pyridazines were developed to inhibit *Escherichia coli* DHPS (EcDHPS) by Burroughs Wellcome Co. (London, UK)^[11] and a decade later various monocyclic 6-(alkylamino)-5-nitrosoisocytosines, including 6-methylamino-5-nitrosoisocytosine (**1**, Figure 1), were published by Lever et al.^[12] Both compound series were pterin-site inhibitors as verified through recent crystallographic studies with *Bacillus anthracis* DHPS (BaDHPS) by the White group.^[7c, 13] The same group also identified other pterin-like compounds, such as 8-mercaptoguanine (8MG) (Figure 1B) and several of these were crystallized in the pterin site.^[13-14]

We have been generating focused libraries based around the 8MG core, having shown that the parent compound inhibits 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK, the enzyme preceding DHPS in the folate pathway), through binding at the equivalent pterin site.^[15] Our recent study demonstrated that some of our 8MG derivatives also bound to EcDHPS,^[15c] which supports the rationale that suitably designed pterin-like analogues may elicit beneficial polypharmacological activity.^[16] Here, we have extended our 8MG chemical probe series and measured equilibrium binding constants using SPR. We report an array of crystal structures comprising novel compounds in complex with EcDHPS that delineate key intermolecular interactions and rationalize SAR of the compound series. Sub-micromolar affinities were observed (**62**, $K_D = 390$ nM, Table 5), including one compound that was shown to bind closely within the substrate envelope (**59**, $K_D = 650$ nM, Table 4), constituting a 200-fold improvement in affinity and a rational evolution of the parent fragment, 8MG.

Results and Discussion

Crystallization of EcDHPS. Initially, protein crystallization trials were performed using Tris-HCl (pH 8.5) as the protein buffer, however poor reproducibility of crystal growth, suspected to arise from stability issues, led us to perform a differential scanning fluorimetry (DSF) assay^[17] to screen for more suitable buffer conditions. A buffer solution containing 50 mM trisodium citrate, 150 mM NaH_2PO_4 , and 350 mM K_2HPO_4 , pH 6.9, was thus determined to provide EcDHPS with markedly improved stability (Figure S4), at both low and high temperatures, over the previously reported conditions,^[7a, 18] which enabled consistent crystal growth and good diffraction for a range of complexes.

8MG is a scaffold hop of the pterin substrate. Previously, 8MG was crystallized in BaDHPS, despite not showing any enzyme inhibition (at 250 μM) in a radiolabelled pABA assay.^[13] 8MG is a small fragment, binding with respectable ligand efficiency (LE) to EcDHPS ($K_D = 76$ μM over 12 heavy atoms, corresponding to a LE of 0.46 kcal·mol⁻¹·heavy atom⁻¹).^[15c] It represents a close scaffold hop from the pterin core^[15a] sharing the same isocytosine ring and a synthetically useful mercapto-group facilitating extension from the 8 position. Elaboration at this position is justified by the fact that the C⁸-S⁸ vector points out from the deep pterin pocket in DHPS (Figure 2), but is also oriented $\sim 30^\circ$ away from the methylene linker of the native substrate. While direct derivatization of the pterin scaffold has been carried out in earlier work (vide supra), elaboration of 8MG has not been investigated as a means to generate pterin-site inhibitors of DHPS.

Figure 2. X-ray crystal structures looking into the pterin pocket of EcDHPS bound to (A) **7** or (B) **1**. Key residues are displayed in green. A water molecule is displayed as a red sphere. Hydrogen bonds from the ligand are displayed as dashed black lines

Functionalization of 8MG at N⁷, N⁹, and C⁸. Prior to derivatization from the S⁸ handle, we briefly probed the chemical space from the N⁷, N⁹, and C⁸ positions. Both the N⁷ and N⁹ positions looked to be rather poor choices for elaboration of the 8MG core from simple superposition of 8MG on the pterin moiety in the EcDHPS structure. For N⁹, the equivalent nitrogen group of the pterin substrate participates in a hydrogen bond with residue D96.^[7a] Methylation at this position (and therefore removal of this interaction), as per **1** (Figure 2) and other inhibitors,^[7c, 13-14] is tolerated, however there appears to be limited space to extend this substituent further. To test this, four

simple 8MG derivatives with either methyl (**3**), ethyl (**4**), benzyl (**5**), or phenethyl (**6**) N⁹-functionalization were tested by SPR (Table 1). Indeed, only methyl-substitution maintained affinity for EcDHPS compared to 8MG (**3**, $K_D = 70 \mu\text{M}$), while the other compounds returned K_D values of 420 μM , 350 μM , and 160 μM , respectively. A crystal structure of EcDHPS/9-methylguanine (**7**, $K_D = 58 \mu\text{M}$) (Figure 2) shows that the methyl group is accommodated by the slight displacement of the catalytic residue D96 — a similar displacement is observed in the solved complex of EcDHPS/**1** — while the remaining hydrogen bonding network of the pterin substrate core is retained (residues N115, D185, and K221, and a water molecule, Figure 2). These structures suggest that any further extension from the N⁹ position would be to a first approximation disfavored by potential close contacts to the D96 residue. However, the affinity trend for these compounds appears to indicate that, as the N⁹ substituent further increases in size, additional interactions to the protein may be formed that could compensate for these unfavorable contacts. Inspection of the EcDHPS structure reveals that extension from the N⁷ position is predicted to be energetically disfavored, since the N⁷ atom of 8MG is part of a bifurcated hydrogen bonding network involving the sidechain of residue K221 and the carbonyl of 8MG.

Table 1. Binding of N7 and N9 8MG derivatives as determined by SPR

compd	X	Y	K_D EcDHPS (μM)
8MG	SH	H	76.2 ± 12^b
2	SCH ₃	H	330^a
3	SH	Methyl	70 ± 9
4	SH	Ethyl	420^a
5	SH	Benzyl	350^a
6	SH	CH ₂ Benzyl	160^a
7	H	Methyl	58 ± 8
8	Br	H	42^a
9	Methyl	H	71^a
10	OH	H	200^a
11	OH	Methyl	101 ± 2
12	N-morpholino	H	160^a

[a]n=1. [b] Dennis et al.^{15c}

Guanine analogues with various functionalities at the C⁸ position — bromo (**8**), methyl (**9**), hydroxyl (**10**), and morpholino (**12**) — were tested (Table 1). Of these, only **8** showed improved binding relative to 8MG (~2-fold), returning an affinity of 42 μM . N⁹-methylation of **10** improved binding 2-fold (**11**, $K_D = 100 \mu\text{M}$);

apart from the direct interactions from the introduced substituent, N⁹-methylation potentially improves binding by more favorable desolvation as well as promoting the bound tautomer, i.e. an unsaturated N⁷-C⁸ bond in which the resulting HBA at N⁷ interacts with the sidechain of K221. As extension from N⁹ showed limited potential, strategically S⁸ represented the obvious group for elaboration, offering synthetic ease for a range for chemical extensions. We therefore focused on functionalization at the S⁸ position.

Table 2. Binding of 8MG-benzyl derivatives as determined by SPR

compd	R	K_D EcDHPS (μM)
13	2-fluoro	8.5 ± 2.1^a
14	2-chloro	7.0 ± 0.8
15	2-bromo	14.0 ± 0.2
16	2-methyl	9.5 ± 0.6
17	2-cyano	12.7 ± 0.3
18	2-nitro	33 ± 2
19	3-methyl	11 ± 2
20	3-methoxy	7.5 ± 0.5
21	3-cyano	11 ± 2
22	4-fluoro	11 ± 2
23	2,2-difluoro	14 ± 3
24	2,4-difluoro	12 ± 3
25	3,4-difluoro	11 ± 1
26	2-fluoro,3-methyl	31 ± 8
27	2-methyl,4-fluoro	13 ± 1
28	3-methyl,4-fluoro	8.2 ± 0.9
29	2,3-dimethyl	14 ± 2
30	2,5-dimethyl	11.5 ± 0.1
31	3,5-dimethyl	14 ± 3

[a] Dennis et al.^{15c}

A variety of substituents on the S⁸ benzyl group are broadly tolerated. Previously we demonstrated that derivatization from the S⁸ position can show improvement over the affinity of 8MG to EcDHPS ($K_D = 76 \mu\text{M}$) with benzyl functionalization yielding binding constants as low as 8.5 μM (compound **13**).^[15c] Here, further substitution from the benzyl ring was investigated (Table 2).

Figure 3. X-ray crystal structures of EcDHPS bound to (A) **13**, (B) **36**, or (C) **41**. Key residues are shown (green) and hydrogen bonds to residue R255 in dashed black lines. Both monomers in the EcDHPS/**13** complex is shown.

Testing with small substituents at all positions (ortho, meta, para), in addition to naphthyl (**32**, **33**), phenylethynyl (**34**), and pyridyl (**35**) analogues, revealed affinities between 7 and 16 μM for 21 of the 22 compounds tested (Tables 2 and 3). The 8MG-benzyl derivative possessing *o*-chloro substitution (**14**) bound with the highest affinity of these compounds. While the SAR is rather broad, these analogues maintain increased binding compared with the simple unsubstituted benzyl ($K_D = 44 \mu\text{M}$) previously measured by SPR.^[15c]

Table 3. Binding of 8MG-benzyl analogues as determined by SPR.

compd	R	K_D EcDHPS (μM)	K_D EcHPPK (μM) ^a	
32^b		15 ± 3	7.3 ± 0.6	
33		16 ± 2	1.46 ± 0.04	
34		9.8 ± 0.9	nd	
35		15 ± 1	15.9 ± 0.1	

[a] In presence of 10 mM Mg^{2+} and 1mM ATP. [b] 84% pure.

A crystal structure of EcDHPS in complex with compound **13** featuring an *o*-fluorobenzyl group, was solved to 1.84 Å resolution (Figure 3A). Two protomers are present in the asymmetric unit, contrasting with the one protomer observed in

the three previously published EcDHPS crystal structures^[7a] where the dimer interface was present through crystallographic symmetry. The new complex is similar, with overall backbone rmsd values of $\sim 0.5 \text{ \AA}$, when compared to these three previous EcDHPS structures (1AJ2, 1AJ0, and 1AJZ).^[7a] The biggest differences between the two monomers of the **13**/EcDHPS complex are for residues 57–70 (loop 2) and 143–151 (loop 5, absent in monomer B). Loop 1 (residues 25–34) is also disordered for both monomers. A key difference between the pterin-binding sites of the monomers is found in the orientation of catalytic residue D96. This interacts with the ligand in monomer A, but is instead oriented towards residue D56 in monomer B — a trend consistent for most of the crystal structures in this study.

The structure shows the 8MG core mimicking the hydrogen bonding network of the substrate. While electron density was strong for the 8MG moiety it was markedly weaker for the benzyl moiety (Figure S2) and its orientation is somewhat ambiguous, although the benzyl moiety appears to interact with residues F190 and K221. The weak density for this group is indicative of mobility and an absence of specific interactions, which may help to explain the broad SAR observed for the 8MG-benzyl series. The site beyond the 8MG-binding pocket is rather open (depending on loop conformations) such that S^8 -functionalized derivatives with appropriate flexibility can generally avoid clashes with the enzyme.

S^8 -methylenecarbonyl derivatives exhibit improved binding. 8MG-acetophenone derivatives, for example **36** ($K_D = 3.9 \mu\text{M}$ ^[15c]), were previously shown to display higher affinities for EcDHPS than 8MG (Table 4).^[15c] The acetyl derivative also showed improved affinity (**38**, $K_D = 7.8 \mu\text{M}$ ^[15c]) relative to 8MG, suggesting that the carbonyl group was primarily responsible for the increased potency. Several amidic compounds (**41–45**) with alkyl *N*-substitution were therefore tested (Table 4, Table S3), a simple *N*-methylene acetamide group (**41**, $K_D = 4.5 \mu\text{M}$) proving the most beneficial. Extending off the methylene linker between the S and amide group was found to significantly reduce affinity (**45**, $K_D > 200 \mu\text{M}$).

X-ray structures of EcDHPS bound to **36** and **41** reveal a hydrogen bond between the carbonyl group of these compounds and the guanidinium group of R255 (Figure 3). The amide of **41** and ketone of **36** overlay well and are oriented approximately perpendicular to the plane of the guanidinium group. The phenyl ring of **36** makes a favorable van der Waals interaction with the alkyl sidechain of K221, though it should be noted that electron density is noticeably poorer for this portion of the molecule,

possibly indicating mobility. The *p*-brominated variant of **36**, compound **37**, bound with a K_D of 2.0 μ M. These two crystal structures indicated that further extension from the amide of **41** may pick up additional interactions to the nearby residues, particularly K221 and F190. 11 compounds (**46–56**) with hydrophobic substituents, and either amide or ester linkers, were thus synthesized. An *N*-phenyl acetamide bound the tightest of these (**54**, K_D = 2.1 μ M). Again, extension from the methylene linker was observed to result in a loss of affinity (cf. **46** and **47**, K_D = 4.5 and 22 μ M, respectively).

Table 4. Binding of 8MG derivatives as determined by SPR

compd	R	K_D EcDHPS	K_D EcHPPK	K_D SaHPPK
		(μ M)	(μ M) ^e	(μ M) ^e
36		3.9 $\pm$ 0.2 ^{ac} (4.3 $\pm$ 0.2 ^d)	0.82 $\pm$ 0.04 ^b	0.60 $\pm$ 0.04 ^b
37		2.0 $\pm$ 0.1	0.70 $\pm$ 0.01 ^b	0.57 $\pm$ 0.01 ^b
38		7.8 $\pm$ 1.0 ^a	nd	1.85 $\pm$ 0.07 ^a
39		18 $\pm$ 4 ^c (11.8 $\pm$ 0.4 ^d)	89.2 $\pm$ 0.5 ^b	7.6 $\pm$ 0.2 ^b
40		20.0 $\pm$ 0.5	68 $\pm$ 1 ^b	4.6 $\pm$ 0.3 ^b
41		4.5 $\pm$ 0.1 ^c (7.6 $\pm$ 0.3 ^d)	50.0 $\pm$ 0.1 ^b	8.8 $\pm$ 1.0 ^b
42		8.2 $\pm$ 0.1	53 $\pm$ 10 ^b	1.5 $\pm$ 0.1 ^b
43		7.5 $\pm$ 0.2	59.4 $\pm$ 0.4 ^b	13 $\pm$ 1 ^b
44		6.9 $\pm$ 0.1	74.2 $\pm$ 0.1 ^b	11 $\pm$ 1 ^b
45		> 200	120 $\pm$ 10 ^b	46 $\pm$ 4 ^b
46		4.53 $\pm$ 0.03	26 $\pm$ 3	2.8 $\pm$ 0.2

47		22 $\pm$ 2	nd	nd
48		7.1 $\pm$ 0.6	nd	nd
49		2.9 $\pm$ 0.1	42 $\pm$ 2	2.6 $\pm$ 0.1
50		5.90 $\pm$ 0.04	62 $\pm$ 7	2.8 $\pm$ 0.7
51		4.08 $\pm$ 0.01	41.8 $\pm$ 0.1	2.20 $\pm$ 0.01
52		4.9 $\pm$ 0.2	66 $\pm$ 1	4.7 $\pm$ 0.3
53		9.6 $\pm$ 0.1	64 $\pm$ 8	4.7 $\pm$ 0.4
54		2.05 $\pm$ 0.02	12 $\pm$ 1	1.4 $\pm$ 0.3
55		12.1 $\pm$ 0.1	67 $\pm$ 1	4.7 $\pm$ 0.8
56		9.9 $\pm$ 0.6	57 $\pm$ 1	4.0 $\pm$ 0.5
57		7.9 $\pm$ 0.3	nd	nd
58		0.83 $\pm$ 0.01 ^c (0.86 $\pm$ 0.01 ^d)	nd	nd
59		0.65 $\pm$ 0.03	> 200	2.7 $\pm$ 0.1
60		4.6 $\pm$ 0.1	8.8 $\pm$ 0.5	12 $\pm$ 3

[a]Dennis et al.¹⁵⁶ [b]Dennis et al.^{15d} [c]Determined using Biacore T200 or [d] ProteOn XPR36. [e] Tested in presence of 10mM Mg²⁺ and 1 mM ATP.

Figure 4. (A) Overlay of both monomers from the X-ray crystal structure of **58**/EcDHPS. X-ray crystal structure of the **59**/DHPS complex, monomer (B) A and (C) B. Key residues are displayed in orange and green, and the ligand is displayed in purple and yellow, for monomer A and B, respectively. Hydrogen bonds to residue R255 are displayed as dashed black lines.

Substituting a secondary amide for a carboxylic acid gave a marked improvement in affinity (**58**, $K_D = 830$ nM). Addition of an extra methylene unit between the sulfur atom and carboxylic acid group (**57**) reduced binding by 10-fold. Aryl substitution at the methylene linker of **58** maintained affinity (**59**, $K_D = 650$ nM), contrasting with the effect of adding a methyl substituent to the methylene linker in the *N*-methyl acetamide-functionalized compounds, **45** and **47**. Crystal structures of EcDHPS in complex with **58** and **59** were solved, revealing that the carbonyl groups in the carboxylic acid moieties are positioned similarly to the carbonyl groups within the **36**- and **41**-bound structures, and that the benzyl group of **59** projects towards loop 2 (Figure 4). In the **59**-bound complex, the interacting loop 2 residues differ between the monomers, with the key residues being T62 for monomer A and R63/P64 for monomer B. The racemic form of **59** was synthesized and tested, however only the (*R*)-enantiomer was present in the X-ray structure, suggesting that the affinity reported in Table 4 is underestimated up to 2-fold. The (*S*)-enantiomer would likely clash with residues F190 and K221. The interactions involving the carboxylic acid groups in these two complexes are not dissimilar to those observed for the carboxylic acid-functionalized pterin analogues reported previously by Zhao et al.^[14]

As discussed above, methylation at the N^9 position of the 8MG core was hypothesized to improve binding for this compound series. Two of the top compounds, **58** and **54**, were therefore methylated to give **61** and **62**, respectively. A respective 2-fold and 5-fold improvement to binding was observed, over their desmethyl equivalents, with K_D values of 420 nM and 390 nM (Table 5). Two 8MG-benzyl derivatives were also N^9 -methylated, however binding was marginally worse than the majority of the 8MG-benzyl series (**63**, $K_D = 19$ μ M, and **64**, $K_D = 30$ μ M). N^9 -methylation of these inhibitors may, therefore, not be generally applicable, or may be specific to the S^8 -functionalization present. A crystal structure of EcDHPS solved in complex with **61** largely resembles the equivalent **58**-bound structure. In the **58**-bound structure (Figure 4), the N^9 nitrogen interacts with either D96 or a water molecule, in monomer A or B, respectively. In the **61**-bound EcDHPS (Figure

5), the N^9 methyl group displaces these interactions, with D96 interacting instead with D56. In the **62**-bound EcDHPS crystal structure (Figure 5), D96 is likewise displaced, but is oriented away from D56, instead interacting with residue T62. In the **62**/EcDHPS complex, the amide carbonyl of **62** interacts with R255 as per the previous structures, while the *N*-phenyl moiety projects up to interact with residues R220, K221, and H257.

Table 5. N^9 -methylated 8MG derivatives

compd	R	K_D EcDHPS (μ M)
61		0.42 ± 0.04
62		0.39 ± 0.08
63		19 ± 4
64		30 ± 10

Figure 5. X-ray crystal structure of EcDHPS bound to (A) **61** or (B) **62**. Hydrogen bonds are displayed as dashed black lines.

A crystal structure of EcDHPS solved in complex with **61** largely resembles the equivalent **58**-bound structure. In the **58**-bound structure (Figure 4), the N⁹ nitrogen interacts with either D96 or a water molecule, in monomer A or B, respectively. In the **61**-bound structure (Figure 5), the N⁹ methyl group displaces these interactions, with D96 interacting instead with D56. In the **62**-bound EcDHPS crystal structure (Figure 5), D96 is likewise displaced, but is oriented away from D56, instead interacting with residue T62. In the **62**/EcDHPS complex, the amide carbonyl of **62** interacts with R255 as per the previous structures, while the *N*-phenyl moiety projects up to interact with residues R220, K221, and H257.

The pteric acid and sulfa conjugate complexes. To further our understanding of the binding site envelope and key interactions within EcDHPS, we crystallized and solved (2.04 Å) the structure of EcDHPS complexed with pteric acid, the oxidized analogue of the catalysis product (Figure 6A). A single pteric acid molecule is bound in the active site for each monomer, although the quality of electron density differs (Figure S2). The binding of pteric acid is similar to that observed in the equivalent *Yersinia pestis*,^[3] *Bacillus anthracis*,^[7c] and *Coxiella burnetii*^[7f] complexes. The pterin moiety hydrogen bonds to residues D96, N115, D185, G217, and K221 while the pABA moiety interacts with the sidechains of F190 (tyrosine in *C. burnetii*) and K221, and the carboxylate hydrogen bonds with the backbone and sidechain of S222 (Figure 6).

Figure 6. X-ray crystal structure of EcDHPS bound to (A) pteric acid or (B) **60**. Both conformations of R63 in the **60**/EcDHPS complex are displayed. Hydrogen bonds to S222 are displayed as dashed black lines.

Recently, a series of pterin-sulfa conjugates were reported, with one of these crystallized in the binding site of *Yersinia pestis* DHPS.^[19] To probe the potential for 8MG to act as a core from which to develop equivalent sulfa conjugates, we used the above pteric acid/EcDHPS complex as a guide for our design. Compound **60** ($K_D = 4.6 \mu\text{M}$), incorporating an ethylene linker between 8MG and a *p*-phenylsulfonamide moiety, was synthesized and then crystallized with EcDHPS. This compound was found to bind as predicted — the phenyl ring forms hydrophobic interactions with F190 and K221, in addition to loop 2 residues, while the sulfonamide group hydrogen bonds to the backbone and sidechain S222 (Figure 6B). Strong electron density is observed for the entire ligand.

Relationship to the substrate envelope. Superposition of the 11 X-ray structures onto the volume occupied by the transition state bound structure^[3] (green) is shown in Figure 7. As anticipated, the pterin-like 8MG, the pteric acid, and the 8MG-conjugate sit closely within the envelope as described by the substrate ligands in the transition state structure. The acetophenone sidechain of compound **36** ($K_D = 3.6 \mu\text{M}$) projects out from the volume occupied by the envelope as does the related but higher affinity **62** ($K_D = 0.39 \mu\text{M}$). In contrast, the smaller and simpler acid derivative (**58**, $K_D = 0.83 \mu\text{M}$) resides within the substrate envelope as expected based on its size. Notably, the larger phenyl analogue, compound **59**, not only maintained potency ($K_D = 0.65 \mu\text{M}$) but has good shape complementarity to the substrate envelope and suitably positioned for further analoguing to engage the phosphate subsite.

Figure 7. Two views showing the positions of the 8MG analogues as observed in the EcDHPS X-ray structure relative to the substrate (green) and the envelope in the transition state structure of DHPS from *Y. pestis* (PDB: 3TYZ). A single magnesium atom (lime green) and a sulfate (orange) are shown from the pteric acid product. Two compounds (**62**, **36**, pink) project outside the substrate envelope whereas **59** (cyan) is confined largely within the volume occupied by the substrate.

Dual DHPS/HPPK binding. Consultation of Tables 3 and 4 indicates that the acetophenone series of 8MG analogues, such as **36** and **37**, offers robust (sub micromolar) binding to both HPPK from *E. coli* and *S. aureus*.^[15c] Previously we showed that the mode of binding of this series to the *S. aureus* HPPK enzyme was very similar to that observed in the *E. coli* enzyme accounting for the remarkably similar affinity.^[15c] In this, the *p*-methoxy or *p*-bromo group projected out of the active site pocket into the solvent and therefore further extension from this position had little effect on affinity. In comparison, the benzyl series of 8MG analogs bound far tighter to SaHPPK by inducing a cryptic binding pocket, specific to the *S. aureus* enzyme. Combined with the structural data presented here, future structure based design of the acetophenone series may therefore have the potential for dual *E. coli* HPPK/DHPS inhibition. Alternatively, **59** shows tight binding to EcDHPS and good binding to SaHPPK, with a 100-fold selectivity to SaHPPK over EcHPPK.^[15d] As mentioned earlier, sequence and structural conservation over the pterin site in DHPS is high and a superposition of the EcDHPS/**59** complex with the deposited SaDHPS structure (Midwest Centre for Structural Genomics, to be published) shows essentially

equivalent intermolecular interactions (Figure 8). For example the position of R255 is exactly superimposable and would make the key interaction between the guanidinium group and to the carboxylic acid of **59**. Notably, as **59** is also confined within the substrate envelope of DHPS, further structure based inhibitor design of SaHPPK in parallel may produce dual enzyme, envelope-compliant, pterin-site inhibitors in *S. aureus*.

Figure 8. Superposition of **59**/EcDHPS (cyan) and SaDHPS (grey) (PDB:4HB7) showing the close sequence and structural similarity in the active site region. Selected hydrogen bonds are shown in pink and equivalent binding site residues are labelled for EcDHPS.

Conclusions

8MG is a pterin-like fragment that binds DHPS projecting a thiol group as a convenient reaction handle for structural elaboration, allowing for the efficient generation of derivatives. We have presented and structurally characterized a number of novel, S⁸-functionalized 8MG derivatives that possess sub-micromolar affinities for EcDHPS constituting a gain of over two orders of magnitude in potency while maintaining an admirable ligand efficiency (0.5 kcal·mol⁻¹·heavy atom⁻¹) of the parent. Characterizing the structure of tight binding novel compounds to the pterin site is an important aspect of our approach and our efforts provide a detailed structural framework to guide further studies aimed at increasing affinity whilst guiding substrate envelope compliance. While structural data revealed that some of our better compounds projected out from the envelope, gratifyingly some of our best compounds reside within the volume occupied by the substrate. These offer considerable promise for further rational elaboration into DHPS inhibitors in a range of species with the potential for dual HPPK/DHPS activity. While we have observed encouraging inhibition in an *E. coli* K-12 minimal media based assay^[20] (data not shown), antimicrobial activity was achieved in the presence of sub-MIC levels of colistin acting as a membrane-permeating/depolarizing agent. This is not unexpected given the polar nature of the guanine core and the tight binding of the substrate. As our compounds reach comparable potency to the substrate through

interactions into the pyrophosphate subsite, the 8MG core may have to be synthetically revisited and either modified (or scaffold hopped) in order to reduce its inherently large polar surface area (tPSA ~100 Å²) to improve permeability. Given that a pterin-site, substrate envelope-compliant DHPS inhibitor will reduce the growth of wild-type and sulfonamide-resistant pathogenic strains, be refractory to point mutation-based resistance mechanisms, and potentially target a network of other folate synthesis enzymes, our 8MG analogues present interesting scaffolds towards targeting the pterin site of this well-validated target.

Experimental Section

Protein preparation, differential scanning fluorimetry, crystallization and deposited PDB ID codes, SPR, chemical synthesis and characterization are reported in the supplementary information.

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FULL PAPER

Within the envelope: Structure base evolution of 8-mercaptoguanine derivatives leads to tight binding to the antibiotic target DHPS and substrate envelope compliance.

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8-Mercaptoguanine derivatives as inhibitors of dihydropteroate synthase

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