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Pterin–sulfa conjugates as dihydropteroate synthase inhibitors and antibacterial agents

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ABSTRACT

The sulfonamide class of antibiotics has been in continuous use for over 70 years. They are thought to act by directly inhibiting dihydropteroate synthase (DHPS), and also acting as prodrugs that sequester pterin pools by forming dead end pterin–sulfonamide conjugates. In this study, eight pterin–sulfonamide conjugates were synthesized using a novel synthetic strategy and their biochemical and microbiological properties were investigated. The conjugates were shown to competitively inhibit DHPS, and inhibition was enhanced by the presence of pyrophosphate that is crucial to catalysis and is known to promote an ordering of the DHPS active site. The co-crystal structure of *Yersinia pestis* DHPS bound to one of the more potent conjugates revealed a mode of binding that is similar to that of the enzymatic product analog pteroic acid. The antimicrobial activities of the pterin–sulfonamide conjugates were measured against *Escherichia coli* in the presence and absence of folate precursors and dependent metabolites. These results show that the conjugates have appreciable antibacterial activity and act by an on target, anti-folate pathway mechanism rather than as simple dead end products.

Sulfonamides were the some of the first successful antimicrobial agents and have been used continuously since the 1940s to treat a wide variety of Gram-positive, Gram-negative and protozoal infections.[1–3] Sulfonamides are usually prescribed in combination with the dihydrofolate reductase inhibitor trimethoprim to maximize therapeutic response and reduce the development of resistance.[4,5] Today, this drug combination is relied upon as a valuable therapeutic option in two important areas: (1) as an inexpensive oral treatment for community acquired bacterial infections resistant to frontline agents such as penicillins and fluoroquinolones; and (2) as a prophylaxis treatment for the prevention of *Pneumocystis jirovecii* pneumonia in immuno-compromised cancer and HIV patients.[6,7] Over time, sulfonamide use has been compromised by the emergence of resistance and the poor tolerance of patients to long-term treatment.[6,8]

Sulfonamides target the enzyme dihydropteroate synthase (DHPS) and mimic the substrate *p*-aminobenzoic acid (*p*ABA).[1,9] We previously reported detailed structural, biochemical and computational studies on the catalytic mechanism of DHPS.[10] DHPS catalyzes the conjugation of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) with *p*ABA to form 7,8-dihydropteroate, an intermediate in the folate biosynthesis pathway (Fig. 1A).[10] In addition to inhibiting DHPS, sulfonamides can also act as alternative substrates by forming dead end pterin–sulfonamide products, which may also inhibit enzymes later in the pathway (Fig. 1B).[10–12] In this role, the contribution to the overall antimicrobial potency of this class of antibiotics is poorly defined. For the purpose of continuing our studies on the inhibition of DHPS and its therapeutic potential, we sought to chemically synthesize a panel of pterin–sulfonamide conjugates.

Repeating the previously published alkylation based route[13] to these conjugates, we obtained only incorrect isomeric products, challenging the previously ascribed pharmacological properties.[14,15] Consequently, a new synthetic route to rapidly and accurately generate pterin–sulfonamide conjugates was developed. The new conjugates were tested for inhibition of DHPS and co-crystallized with the enzyme to characterize their mode of binding. Microbiological profiling using *Escherichia coli* indicated that pterin–sulfonamides are not only good inhibitors of DHPS but also have substantial antimicrobial activity that can be antagonized by folate precursors.

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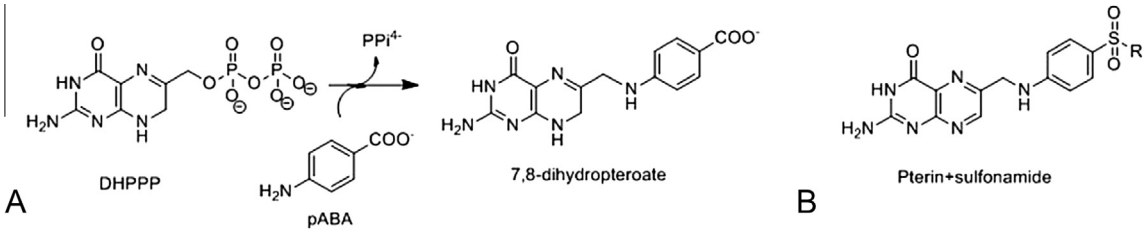
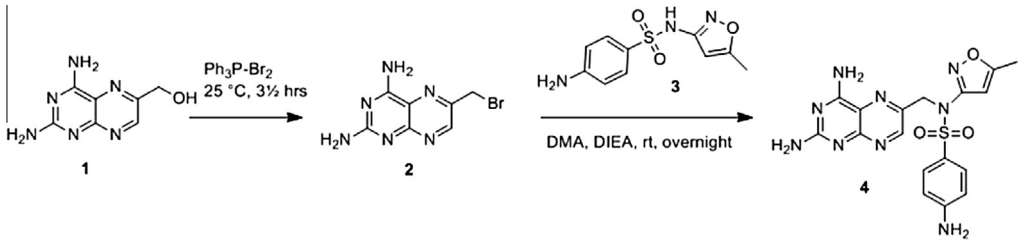
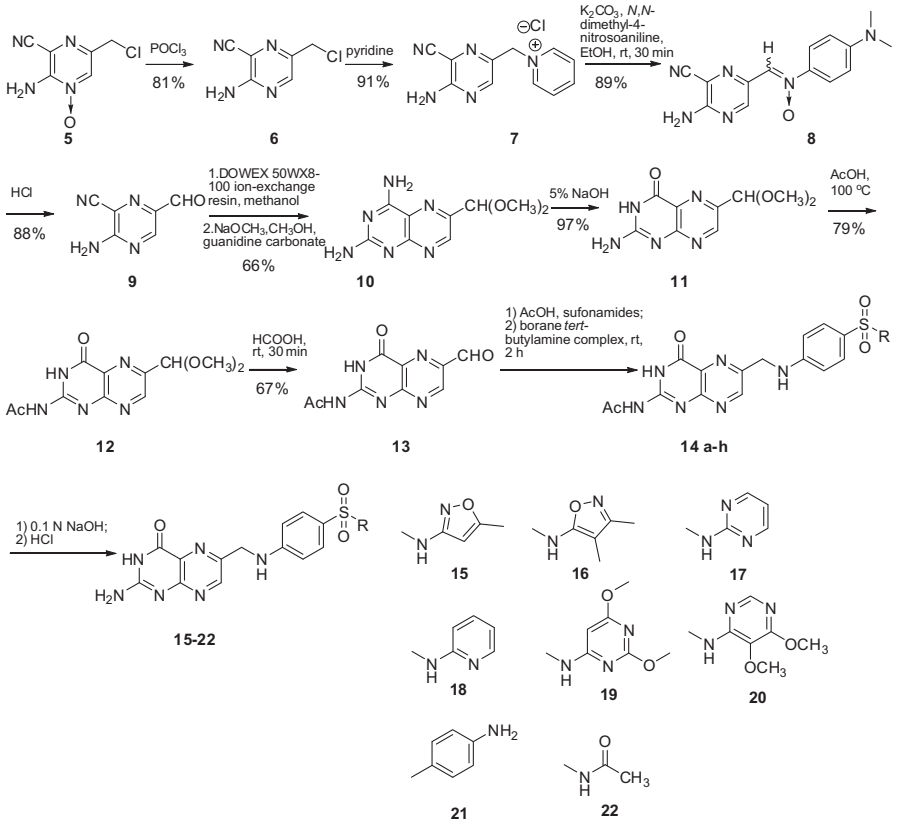


Figure 1. (A) Reaction catalyzed by dihydropteroate synthase; (B) general structure of pterin–sulfonamide conjugates.



Scheme 1. Initial synthetic strategy.

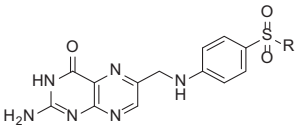


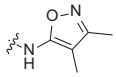
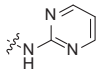
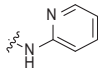
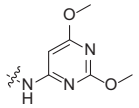
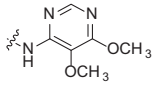
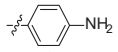
Scheme 2. Synthetic method for production of pterin–sulfonamide conjugates.

Our initial strategy to synthesize the conjugates was based on the method detailed by Piper and Montgomery.[13] Briefly, commercially available (2,4-diaminopteridin-6-yl)methanol **1** was treated with triphenyl phosphine and bromine to afford the 6-bromomethyl pterin analog **2**,[13] which was expected to couple with the appropriate sulfonamide, followed by 4-deamination, to give target compounds in three steps (Scheme 1). However, when compound **2** reacted with sulfamethoxazole **3** in the presence of *N*,*N*-diisopropylethylamine (DIEA), only isomeric sulfonamide linked compound **4** was isolated as the main product, instead of the desired aniline linked pterin–sulfamethoxazole **15**. Attempts to modify the synthesis by protection of the sulfonamide or alter the alkylating conditions failed.[16–18]

An alternative synthetic route that provided the necessary flexibility to conjugate a large variety of sulfonamides to pterin was developed around the key acyl protected formyl-pterin

Table 1
Inhibitory activities of pterin–sulfa conjugates



Compd	R	*Ba*DHPS in vitro FP binding inhibition IC_{50} (μM)		Minimum inhibitory concentration *E. coli* K12, in μM[b,c]		
		(−) PP_i[a]	(+) 2 mM PP_i[a]	M9 media	M9 plus 5 μg/mL PABA	M9 plus 20 μg/mL methionine
15		20.6 ± 3.5	3.4 ± 0.1	10.9 (0.8)[c]	467 (263)[c]	29 (1.5)[c]
16		22.5 ± 1.0	8.1 ± 0.7	28.3 (1.5)	>412 (249)	47 (5.9)
17		19.3 ± 0.6	9.4 ± 0.7	17.1 (1.6)	>470 (666)	78 (6.2)
18		>62.5	2.5 ± 0.2	29.5 (2.7)	>471 (669)	98 (6.3)
19		26.8 ± 3.0	1.9 ± 0.1	17.2 (1.6)	>412 (537)	86 (3.4)
20		15.2 ± 0.6	11.5 ± 0.8	34.3 (3.8)	>412 (>644)	172 (10.1)
21		17.8 ± 0.8	11.3 ± 1.1	197 (25)	236 (>934)	236 (50)
22		18.6 ± 0.8	17.1 ± 1.2	136 (22)	>514 (>934)	>200 (58)
	Ampicillin	ND	ND	1.58	2	2.1
	Chloramphenicol	ND	ND	8.5	9.7	9.7
	Trimethoprim	ND	ND	1.7	1.4	0.7

[a] Present as sodium pyrophosphate. Average of 3 experiments, ±SEM.
[b] Performed according to.[24]
[c] Corresponding values for parent sulfonamides shown in parentheses.

intermediate **13** shown in Scheme 2. Treatment of commercially available 2-amino-5-(chloromethyl)-3-cyanopyrazine 1-oxide **5** with phosphorus trichloride at room temperature in tetrahydrofuran solution resulted in smooth deoxygenation to give **6**,[19] which was treated with pyridine with stirring overnight at room temperature to afford pyridinium salt **7**.[20] Salt **7** reacted with *N*,*N*-dimethyl-4-nitrosoaniline in the presence of potassium carbonate to give nitrone **8**, which was then treated with 6 N HCl to give aldehyde **9**. Quantitative conversion of **9** to its dimethyl acetal in the presence of DOWEX 50WEX8-100 ion-exchange resin, followed by condensation with guanidine carbonate in the presence of sodium methoxide, afforded 2,4-diamino pterin **10**. Treatment of **10** with hot 5% sodium hydroxide gave 6-formylpterindine dimethyl acetal **11**, which was treated with acetic anhydride at 100 °C to give 2-acetyl protected **12**. The 6-formylpterine dimethyl acetal **12** was hydrolyzed with the treatment of formic acid to afford 6-formylpterin **13**.[21] Coupling of 6-formylpterin **13** with different sulfonamides or dapsone (4-[(4-aminobenzene)sulfonyl] aniline) was carried out smoothly in acetic acid to give the corresponding Schiff base compound, which was subsequently reduced with borane *tert*-butylamine complex to give **14a**–**h**.[22] Deprotection of **14a**–**h** with 0.1 N NaOH afforded target compounds **15**–**22**.[23]

Compounds **15**–**22** were all tested for inhibition of *Bacillus anthracis* DHPS using a fluorescence polarization (FP) based competition binding assay, performed in the absence or presence of 2 mM sodium pyrophosphate (Table 1 and Supplementary material).[25] Pyrophosphate (PP_i) is the biochemical byproduct of DHPS catalysis, and its inclusion in the FP assay allowed us to confirm the site-specific binding of pterin–sulfonamide conjugates. More specifically, we have previously observed that PP_i stabilizes two flexible loops in the DHPS active site that form the *p*ABA–sulfonamide binding site.[10]

As a group, the pterin–sulfonamide conjugated compounds are more efficient than SMX (FP IC_{50} > 62.5 μM) at displacing the probe from the active site in the FP competition assay. These compounds show an even more efficient displacement of probe when PP_i is added to the assay. Among all analogs, pterin–sulfadimethoxine (pterin–SDM) conjugate **19** and pterin–sulfamethoxazole (pterin–SMX) conjugate **15** have the highest potency against *Ba*DHPS, with IC_{50} values of 1.9 μM and 3.4 μM, respectively, in the presence of PP_i. Conjugation of pterin with other sulfa analogs resulted in compounds with good inhibitory activity with the weakest inhibition observed for the sulfanilamide conjugate **22**.

To establish the mode of binding of the pterin–sulfonamide conjugates in the DHPS active site, we soaked compound **16** into

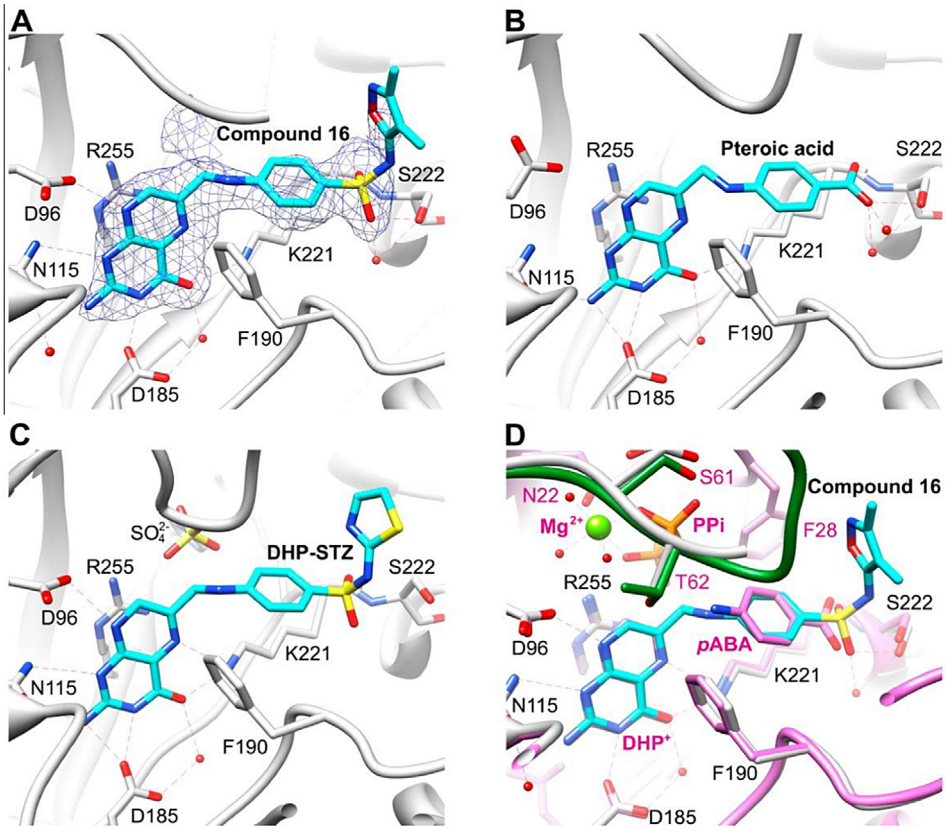


Figure 2. (A) *Yersinia pestis* DHPS (YpDHPS) with compound **16** at the active site. Also shown is the omit map (mFo-DFc) showing the electron density of **16** contoured at 2σ (PDB ID: 5JQ9). (B) YpDHPS with the product analog pteroic acid bound at the active site (PDB ID: 3TYU). (C) *Bacillus anthracis* DHPS (*Ba*DHPS) with the pterin–sulfathiazole (DHP–STZ) catalytic product bound at the active site (PDB ID: 3TYE). (D) Superimposition of the YpDHPS **16** complex and the YpDHPS near transition state complex (PDB ID: 3TYZ). In all figures, the DHPS cartoon and carbon atoms are gray, the oxygen, nitrogen and sulfur atoms are red, blue and yellow respectively, and the compound carbons are cyan. In (D), the transition state structure is shown as pink cartoon and carbon atoms, the ordered loop2 is shown in green, and the essential Mg^{2+} ion is shown in lime.

apo crystals of *Yersinia pestis* DHPS (YpDHPS) and determined the structure of the complex to 2.1 Å resolution (PDB ID: 5JQ9). As anticipated, **16** occupies both the pterin and *p*ABA binding pocket of DHPS (Fig. 2A). The mode of binding is very similar to those of pteroic acid[26] and the sulfathiazole–pterin adduct that was generated catalytically *in crystallo*[10] (Fig. 2B and C). Residues D96, N115, D185, K221 and R255 interact with the pterin moiety, and F190, K221 and S222 interact with the sulfonamide/*p*ABA groups (YpDHPS residue numbering). We have also determined the structure of the DHPS near transition state structure,[10] and its superposition onto the **16** complex shows how closely the compound mimics the binding modes of pterin and *p*ABA. Note that weak electron density adjacent to **16** (Fig. 2A) corresponds to the DHPS anion-binding pocket, which is occupied by sulfate and pyrophosphate in the DHP–STZ and transition state structures, respectively (Fig. 2C and D). Occupancy of the anion-binding pocket by pyrophosphate and the resulting stabilization of loop2 (Fig. 2D) would explain our observation that pyrophosphate augments the binding of the conjugate compounds.

The antibacterial activities of these conjugate compounds and their corresponding parent sulfonamides were tested against *Escherichia coli* K12 (Table 1) in M9 minimal media[27] suitable for testing sulfonamide activity. Under these conditions, most conjugates showed appreciable antibacterial activity, with the exception of the sulfanilamide and dapsone hybrids (**21**, **22**) mirroring the weaker direct activity of the corresponding parent sulfonamides.[24] The sulfamethoxazole hybrid (**15**) showed the most potency with a MIC of 10.9 μM. Overall the minimal inhibitory concentration activities for the conjugates were usually around 10 times weaker than for the corresponding parent sulfonamides (compound **15** 10.9 μM, vs sulfamethoxazole 0.8 μM).

We next explored whether the observed antimicrobial activities are a result of on target biochemical inhibition. MIC activities were examined in the presence of the added folate precursor *p*ABA or the dependent metabolite methionine (Table 1). Addition of *p*ABA completely ablated MIC activity for all conjugates,[28] and a similar but less dramatic effect was seen when growth media was supplemented with methionine. These data mirrored the MIC shifts we observed with the corresponding sulfonamides, supporting the hypothesis that the pterin conjugates function as direct antibacterial compounds through inhibition of the folate pathway.

In conclusion, we have demonstrated that pterin–sulfonamide conjugates have antibacterial action through biochemical inhibition of DHPS and antagonism of the folate pathway. It is important to note that the conjugates synthesized in this study are in the oxidized form of pterin and not the reduced form dihydropterin that is formed by the natural biochemical action of DHPS on DHPPP and sulfonamides, which likely accounts for some of the reduction in pharmacological activity. However, our structures reveal that the oxidation state of the pterin ring has little effect on how it binds into the pterin pocket (compare Fig. 2A and B with C and D).

The lower MIC activity of the conjugates compared to their corresponding sulfonamides may be attributable to differences in penetration and efflux of the pterin sulfonamide conjugates versus sulfonamides. Further studies are ongoing to study the susceptibility to efflux, capacity to inhibit enzymes later in the pathway and

in cell metabolism of the conjugates. The pharmacological advantages that pterin–sulfonamide conjugates may have over sulfonamides are also worthy of further study. The conjugates may have a lower propensity for sulfonamide mediated anaphylaxis, as the oxidatively reactive aniline group in the sulfonamides is hindered by the pterin conjugation.[29] Another important observation from this study is the enhanced binding to DHPS by these conjugates that presumably results from the introduction of a pterin anchor. The pterin anchor also has implications for the development of resistance to these conjugates. As previously noted,[30] the pterin pocket is highly conserved and unlikely to tolerate mutations that might decrease binding affinity. This is not true of the isolated sulfonamides because the *p*ABA-binding site can tolerate such mutations.[10] Thus, the activity of the conjugates against defined sulfonamide resistant strains should be determined.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.07.006.

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23. *General procedure for the synthesis of target compounds:* E.g., 4-((2-Amino-4-oxo-3,4-dihydropteridin-6-yl)methylamino)-*N*-(5-methylisoxazol-3-yl) benzenesulfonamide (**15**): A mixture of *N*-(6-((4-(*N*-(5-methylisoxazol-3-yl)sulfamoyl)phenylamino)methyl)-4-oxo-3,4-dihydropteridin-2-yl) acetamide (0.12 g, 0.255 mmol) in 0.1 M NaOH (24 mL) was stirred at room temperature overnight. The reaction was quenched by adjusting the pH to 8 with diluted HCl. The reaction solution was concentrated to about 5 mL, then the reaction solution was adjusted to pH 5–6 with diluted HCl and filtered, washed with water and MeOH, then dried to give a yellow solid (0.106 g, 97%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 4.46 (d, 2H, J = 5.6 Hz), 6.03 (s, 1H), 6.68, 7.50 (dd, 4H, J = 8.8 Hz), 7.07 (br s, 2H, D_2O exch), 7.33 (br t, 1H, J = 5.6 Hz, D_2O exch), 8.64 (s, 1H), 11.09 (br s, 1H, D_2O exch), 11.57 (br s, 1H, D_2O exch); m/z 429.2 (ES+H+).
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