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Synthesis and Analysis of Bacterial Folate Metabolism Intermediates and Antifolates

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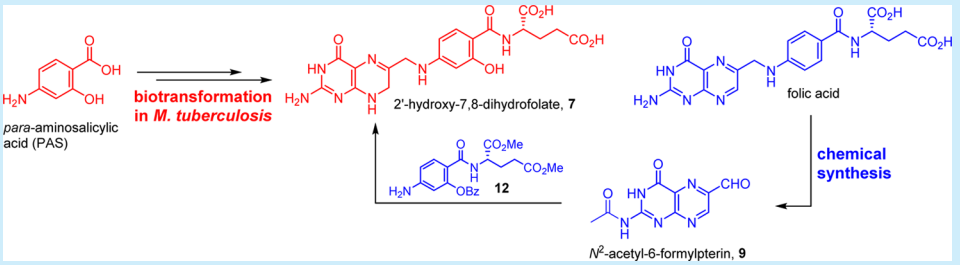
Supporting Information



ABSTRACT: The mechanism of action of *para*-aminosalicylic acid (PAS), a drug used to treat drug-resistant tuberculosis (TB), has been confirmed through the first synthesis and biochemical characterization of its active metabolite **7**. The synthesis features the coupling of N^2-acetyl-6-formylpterin obtained from the degradation of folic acid and appropriately functionalized arylamines to form Schiff bases. The sequential chemoselective reduction of the imine and pterin ring led to the formation of dihydrofolate analogue **7** and two other dihydropteroate species.

Folate species play an essential role in most organisms as cofactors for the biosynthesis of critical cellular components in nucleobases, proteins, and cofactors. Humans lack the *de novo* folate biosynthesis pathway and must obtain this essential nutrient from their diet in the form of the vitamin B9, folic acid. In contrast, most microbes are unable to acquire folates from their external environment and rely on *de novo* folate biosynthesis. The presence of this biosynthetic pathway in microorganisms, but not in humans, makes it an ideal target for antimicrobial drug development. Similar to other bacteria, the *Mycobacterium tuberculosis* folate pathway starts with 6-hydroxymethyl-7,8-dihydropterin **1** (Figure 1, see compound **7** for the numbering of folate atoms), which is obtained from guanosine triphosphate (GTP) through several steps. 6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK) catalyzes the pyrophosphorylation of **1** to form 6-hydroxymethyl-7,8-dihydropterin pyrophosphate **2** employing ATP as the pyrophosphate donor. In the next step, the intermediate **2** is condensed with *para*-aminobenzoic acid (PABA) by dihydropteroate synthase (DHPS, *M. tuberculosis* FolP1) to provide 7,8-dihydropteroate **3**. PABA is produced from chorismate, a central metabolite in the shikimic acid pathway. The following enzyme in the pathway, dihydrofolate synthase (DHFS, *M. tuberculosis* FolC), catalyzes the coupling of **3** and glutamate to afford 7,8-dihydrofolate **4**. Conversion of **4** to tetrahydrofolate **5** is accomplished by dihydrofolate reductase (DHFR, *M. tuberculosis* DfrA), which reduces the 5−6 double bond of the pterin ring. Tetrahydrofolate **5** is subsequently converted to a number of essential cofactors involved in various one carbon metabolism processes.

para-Aminosalicylic acid (PAS)[1] is an important second-line antitubercular agent for the treatment of tuberculosis (TB), but its precise mechanism of action (MOA) has remained elusive. PAS was initially shown to interfere with folate biosynthesis based on its structural similarity to PABA and the observation that the antimycobacterial action of PAS could be fully antagonized by exogenous PABA.[2] Further insight into the MOA was illuminated through metabolomic studies, which demonstrated PAS acts as an antimetabolite and enters the folate pathway by mimicking PABA where it is converted by DHPS to 2′-hydroxy-7,8-dihydropteroate **6** and then by DHFS to 2′-hydroxy-7,8-dihydrofolate **7** (Figure 1). The folate species **7** was hypothesized to inhibit the essential *M. tuberculosis* DHFR enzyme encoded by *dfrA*, thereby causing arrest of folate-dependent metabolism.[3−5] However, this hypothesis has not been biochemically validated with a pure synthetic standard of **7**. A previous attempt to synthesize **7** was unsuccessful, which the authors attribute "to the production of unstable intermediaries".[4]

Herein, we report the first synthesis and biochemical characterization of **7** as well as a concise synthesis of pteroates **3** and **6**. We also describe an analytical liquid chromatography−tandem mass (LC-MS/MS) method for quantitation of all three compounds in complex biological samples as well as the half-lives of these reduced species under aerobic conditions.

The synthesis of 2′-hydroxy-7,8-dihydrofolate **7** (Scheme 1) utilized a convergent strategy instead of a linear approach of

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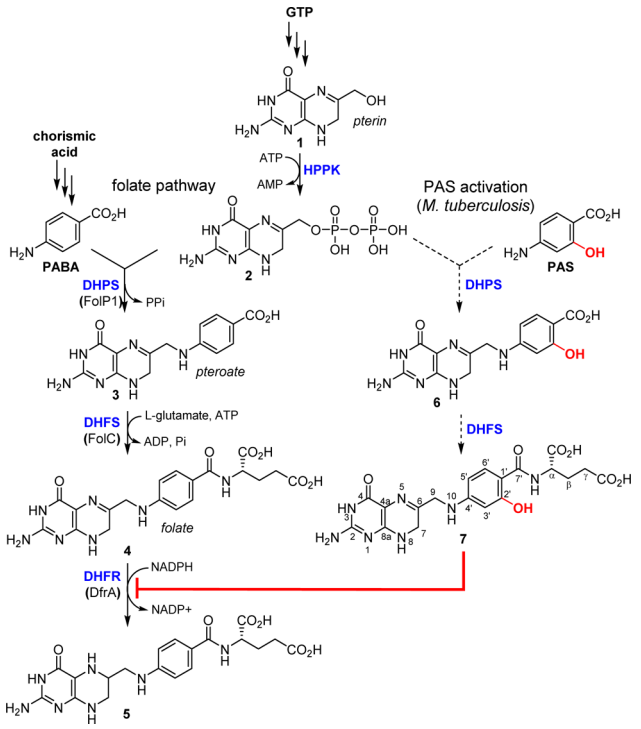
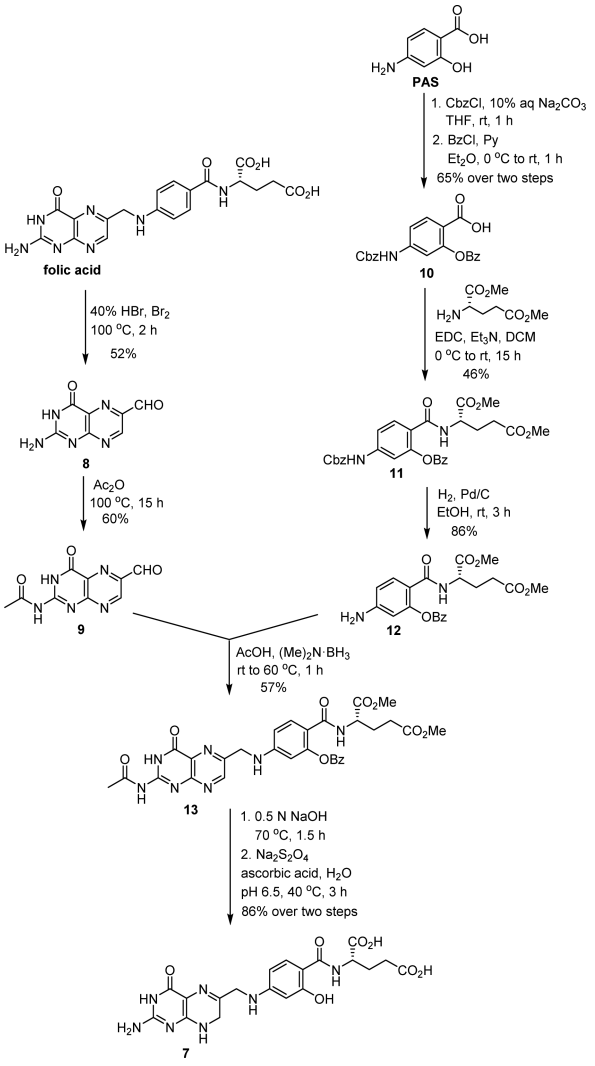


Figure 1. Bacterial folate metabolism pathway (solid arrows) and PAS activation in *M. tuberculosis* (dashed arrows). The name of corresponding *M. tuberculosis* enzyme is provided in parentheses.

converting pterins to pteroates to folates.[6] The synthesis features a coupling between the formylpterin **9** and glutamoylated PAS derivative **12**. The pterin moiety was obtained from the degradation of commercially available folic acid using the method described by Thijssen.[7] A solution of folic acid in 40% aqueous hydrogen bromide and excess bromine was heated at 100 °C to obtain 6-formylpterin **8** in 52% yield (Scheme 1). The reaction proceeds via the benzylic bromination at C-6 of folic acid followed by elimination of HBr to give a Schiff-base intermediate, which rapidly undergoes hydrolysis under the acidic reaction conditions to afford the aldehyde **8**. Among the other reported methods for the synthesis of 6-formylpterin, Thijssen's method was found to be the shortest and most efficient.[8,9] Acetylation of the amino group of **8** was optimally performed in neat acetic anhydride to yield N^2-acetyl-6-formylpterin **9**.[8] The N^2-acetylation was critical to improve the solubility profile of notoriously insoluble[10] pterins and pteroates as well as to prevent possible dimerization in the next synthetic step. Compound **12** was synthesized from PAS in four steps. Cbz protection of amino group of PAS and Bz protection of the phenol provided the carboxylic acid **10**. EDC mediated amide coupling between **10** and dimethyl glutamate afforded **11**, which was converted to the free amine **12** by Pd-catalyzed hydrogenolysis. Reductive amination of **9** and **12** employing dimethylamine·borane in acetic acid furnished **13** that was purified by normal-phase silica-gel column chromatography. Dimethylamine·borane was preferentially employed for this reaction due to its excellent chemoselectivity for the reduction of the intermediate imine over the pterin ring.[11] Finally, one-pot global deprotection and reduction of pterin ring was performed to obtain the product **7**. This was accomplished by simultaneous deacetylation, debenzoylation, and methyl ester hydrolysis of **13** in the presence of 0.5 N NaOH at 70 °C followed by sodium dithionite mediated selective reduction of the 7,8 double bond in the presence of the antioxidant ascorbic acid at pH 6.5 at 40 °C

Scheme 1. Synthesis of 2′-Hydroxy-7,8-dihydrofolate 7



(Scheme 1).[12] The sodium dithionite reduction was slower at rt with approximately 50% conversion at 3 h, but was complete at 40 °C in the same duration. The reduction was conveniently monitored by ^{1}H NMR where the C-7 proton chemical shift moves upfield from 8.8 to 3.8 ppm. This represents the first reported synthesis of PAS derived bacterial folate antimetabolite **7**.

The intermediate N^2-acetyl-6-formylpterin **9** was conveniently employed for the synthesis of the other two reduced folate compounds **3** and **6** (Scheme 2). Using the strategy developed in Scheme 1, Schiff-base formation of aldehyde **9** with ethyl *para*-aminobenzoate **14** and ethyl *para*-aminosalicylate **15**[13] and in situ reduction with dimethylamine borane yielded the ethyl N^2-acetylpteroate **16** and ethyl N^2-2′-hydroxypteroate **17**, respectively. Both products precipitated out of the reaction mixture, and the precipitates were filtered and washed with Et_2O to obtain the pure products **16** and **17**. Deacetylation and ethyl ester hydrolysis of **16** and **17** in the presence of 1 N NaOH at 70 °C followed by sodium dithionite reduction of the pterin ring afforded the metabolites **3** and **6**, respectively. This route provided the first synthesis of antimetabolite **6** and offers a particularly facile synthesis[14] of the natural folate metabolite **3**.

The final products **3**, **6**, and **7** all have very low aqueous solubility at acidic pH and precipitated out when the pH of the

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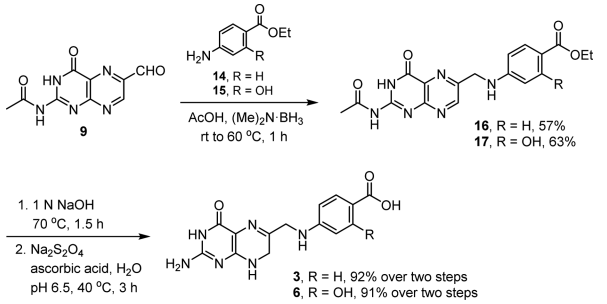
Scheme 2. Synthesis of 7,8-Dihydropteroate 3 and 2′-Hydroxy-7,8-dihydropteroate 6

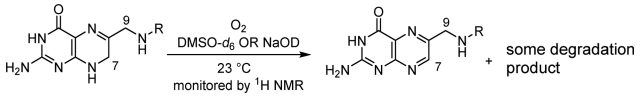


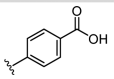
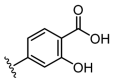
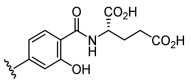
reaction mixture was lowered to ~3. The compounds are stable for months when stored at 4 °C and pH 2 as a precipitated suspension of the reaction mixture which contains about 5% (w/v) antioxidant ascorbic acid. If stored in this manner, the compounds can be rapidly recovered to prepare a DMSO stock solution for immediate biological evaluation as follows: the suspension is centrifuged, and the residue is washed successively by H_2O, EtOH, and Et_2O, then dried with a stream of argon, and dissolved in DMSO. Less than 5% degradation of compound **7** was observed in 6 months when stored as described above. The compounds are also stable when stored as solids (after Et_2O wash) at −80 °C in an argon filled amber vial.

With an authentic sample of **7**, we proceeded to evaluate it for inhibition of recombinant DfrA,[15] the DHFR ortholog in *M. tuberculosis* using a coupled steady-state kinetic spectroscopic assay under initial velocity conditions that measures consumption of NADPH. These experiments confirmed 2′-hydroxy-7,8-dihydrofolate **7** is a potent inhibitor of DfrA with respect to 7,8-dihydrofolate. The inhibition constant (K_i) derived from this analysis using the Cheng−Prushoff equation is 174 ± 102 nM (Figure S1). These results provide the first biochemical validation for the mechanism of action of PAS, which innocently enters the folate pathway exploiting the relaxed substrate specificity of the mycobacterial enzymes DHPS and DHFS that together convert PAS into **7**, that in turn inhibits the downstream enzyme DHFR. This further clarifies our understanding of this old drug first introduced into clinical use nearly 80 years ago.

The naturally occurring reduced folate species are well-known to undergo spontaneous oxidative degradation; however, dihydrofolate species are relatively more stable than tetrahydrofolate species.[16] Reduced folates are also much more prone to air oxidation and degradation at lower pH, but at pH higher than 7 they are quite stable.[17] In order to determine the solution stability of these compounds under air, we studied the kinetics of air oxidation. A 20 mM solution of each compound in DMSO-d_6 or 1.5 N NaOD were exposed to air and incubated at room temperature without exclusion of light, and the rate of oxidation was studied by ^{1}H NMR. The ratios of oxidized and reduced forms were readily calculated by integration of C-7 (oxidized form: ~8.8 ppm, reduced form: ~3.8 ppm) and C-9 protons (oxidized form: ~4.6 ppm, reduced form: ~3.9 ppm) that exhibited diagnostic and well resolved chemicals shifts for each species. Based on NMR, a third compound was also formed over time which was likely the 6-formylpterin.[16] The oxidation followed first-order kinetics, and the half-lives were determined (Table 1). Among the three compounds examined, 2′-hydroxy-7,8-dihydropteroate **6** was the least stable in DMSO possessing a half-life of less than 2 h. The other two compounds, by comparison, exhibited half-lives of more than 5 h under aerobic conditions at ambient temperature.

Table 1. Air Oxidation Half-Lives of 3, 6, and 7 in DMSO-d_6 and 1.5 N NaOD at 23 °C



compound	R	$t_{1/2}$ (h) DMSO-d_6	$t_{1/2}$ (h) NaOD
3		7.9	12.2
6		1.8	18.7
7		5.3	(83)[a]

[a]Percent remaining in 24 h.

All three compounds displayed significantly improved stabilities in 1.5 N NaOD. Thus, only 17% of the compound **7** was degraded in 24 h under these alkaline conditions. Interestingly, DMSO solutions of all three compounds turned dark red within 2 h, whereas NaOD solutions persisted unchanged as light orange solutions.

We next sought to develop a sensitive analytical method employing high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) that could be used for subsequent studies to monitor intracellular levels of the natural folate metabolite **3**, and PAS derived biotransformation products **6** and **7**. As the compounds were more stable under alkaline conditions, a buffer system at pH 9 was employed for chromatography. Additionally, the compounds showed superior ionization in ESI negative mode revealing the $[M-H]^-$ peaks for the parent molecular ions. Representative MS/MS spectra including parent ions and major fragment ions used for multiple reaction monitoring (MRM) are provided in Figure 2A−C. These MS/MS spectra were obtained by direct infusion of 10 μM aqueous acetonitrile (1:1, 10 mM ammonium acetate, pH 9.0) solutions of **3**, **6**, and **7** into the ESI source of the mass spectrometer at a flow rate of 10 μL/min. The major fragmentation pattern observed for pteroates **3** and **6** was due to the cleavage of the C_9-N_{10} bond, which produced PABA (m/z 135.9) and PAS (m/z 151.8) anions, respectively, as well as the pterin anion (m/z 175.9). The majority of fragment ions for hydroxyfolate **7** were obtained from expulsion of the glutamoyl moiety and the cleavage of C_9-N_{10} bond. Next, to determine the extent of matrix associated ion suppression in the biological samples, the standard solutions were prepared by spiking synthetic standards into a mycobacterial extract.[18] We generated a calibration curve for each compound, and the Limits of Quantitation (LOQ) for each compound was identified (<6 ng/mL, Figure 2D). The peak area was linear in the concentration range of LOQ to 0.4 μg/mL with R^2 > 0.99 (Supporting Information) providing a large dynamic range for quantitation. A representative chromatogram is shown in Figure 2, and a detailed method is provided in the Supporting Information.

In summary, we have accomplished the first synthesis of the PAS derived antifolate species **6** and **7** using a convergent approach and shown both species are quite unstable under aerobic conditions, but can be safely stored for months as a suspension with 5% w/v ascorbic acid. We envision that the strategy utilized here of obtaining the formylpterin by the

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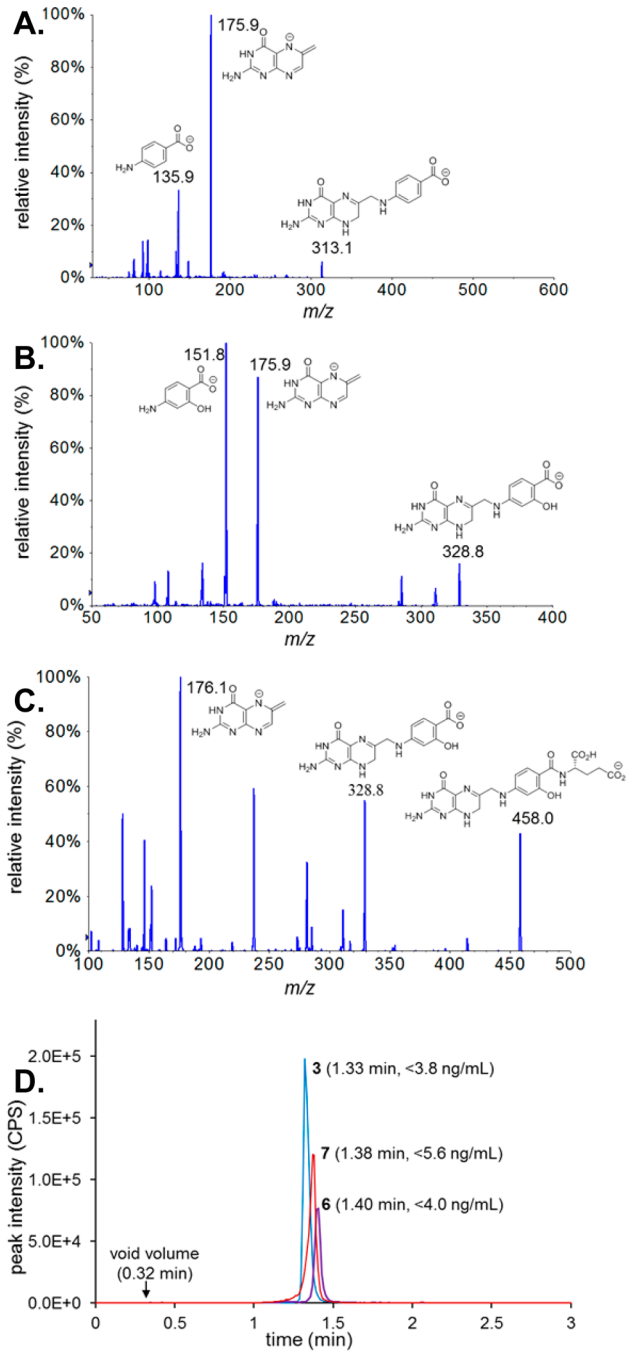

Figure 2. Negative-ion MS/MS fragmentation spectra of (A) 7,8-dihydropteroate **3**, (B) 2′-hydroxy-7,8-dihydropteroate **6**, and (C) 2′-hydroxy-7,8-dihydrofolate **7**. The transformations m/z 313.1 → 175.9 and 313.1 → 135.9 for **3**, 328.8 → 175.9 and 328.8 → 151.8 for **6**, and 458.0 → 328.8 and 458.0 → 176.1 for **7** were used for multiple reaction monitoring (MRM). (D) LC-MS/MS traces of compounds **3**, **6**, and **7** in MRM mode. Retention time and limit of quantitation (LOQ) are provided in parentheses.

chemical degradation of commercial folic acid and coupling it with arylamines may find application in the synthesis of other modified folates and pteroates. We confirmed **7** is a potent inhibitor of *M. tuberculosis* DHFR with a K_i of 174 nM providing biochemical validation for the mode of action of PAS. Finally, we developed a robust LC-MS/MS method for quantitation of these antimetabolites in bacterial cell lysates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.7b02487.

Experimental procedure, biochemical methods, LC-MS/MS method, and ^{1}H and ^{13}C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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