Synthesis and Biological Evaluation of 2-Amino-4-hydroxy-6-hydroxymethylpteridine Pyrophosphate

RICHARD I. HO ˣ, LEONARD CORMAN, and WILLIAM O. FOYE

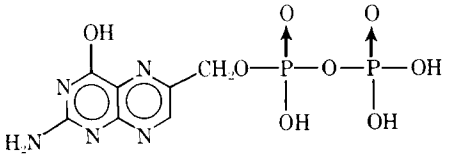
Abstract □ A method for synthesizing the title compound is described which represents a distinct improvement over previous procedures in regard to convenience, yield, and purity. IR and proton NMR spectral data are reported for this compound for the first time. The effectiveness of 2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate as a substrate for the enzyme dihydropteroate synthetase, extracted from both *Neisseria gonorrhoeae* and *N. meningitidis*, was evaluated. Inhibition of this enzyme, using the pyrophosphate as substrate, was observed with sulfa drugs and related compounds.

Keyphrases □ 2-Amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate—synthesis and biological evaluation, effectiveness as substrate for dihydropteroate synthetase □ Dihydropteroate synthetase—effectiveness of 2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate as substrate, effects of sulfa drugs and related compounds □ Enzyme inhibition—dihydropteroate synthetase by sulfa drugs and related compounds using 2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate as substrate

The enzymatic synthesis of dihydropteroate and dihydrofolate involves the coupling of hydroxymethylpteridine pyrophosphate with *p*-aminobenzoate or *p*-aminobenzoylglutamate (1–3). The first synthesis of hydroxymethylpteridine pyrophosphate (2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate) (I) was described by Shiota *et al.* (4, 5), and this method has been employed extensively for studies related to the biosynthesis of pteroate and analogs. This method of synthesis, however, is tedious and results in preparations that are invariably contaminated with simple pteridines, particularly pteridine monophosphate (5). Purification of these products is difficult, and yields are quite low (~5%). A similar synthesis of hydroxymethylpteridine pyrophosphate was reported (6), but it is also unsatisfactory since it yields a variety of phosphorylated pteridines, requiring extensive purification.

DISCUSSION

This article describes an improved synthesis of this compound, which is obtained free of contaminating pteridines; the monophosphate ester is completely absent. Furthermore, this procedure requires only one step, and no further purification is needed beyond recrystallization. In this procedure, an excess of crushed pyrophosphoric acid is melted slowly, and the hydroxymethylpteridine, prepared by the method of Forrest and Walker (7), is added slowly to avoid polymerization. The product is adsorbed on acid-washed active charcoal and eluted rapidly with a carefully controlled amount of ammonium hydroxide solution. If a solvent system of ammonium hydroxide and absolute ethanol is used, as with the Shiota *et al.* (5) procedure, other phosphorylated pteridines appear in the eluate. The eluate is checked for impurities by paper chromatography, using water as the solvent. The desired compound moves with the solvent front; if another spot appears, the extraction is stopped. This chromatographic technique has provided maximum yields and ensured purity of product. The compound is obtained as the diammonium salt, and its purity was substantiated by passage of the eluate through a column[1]; a single peak resulted.



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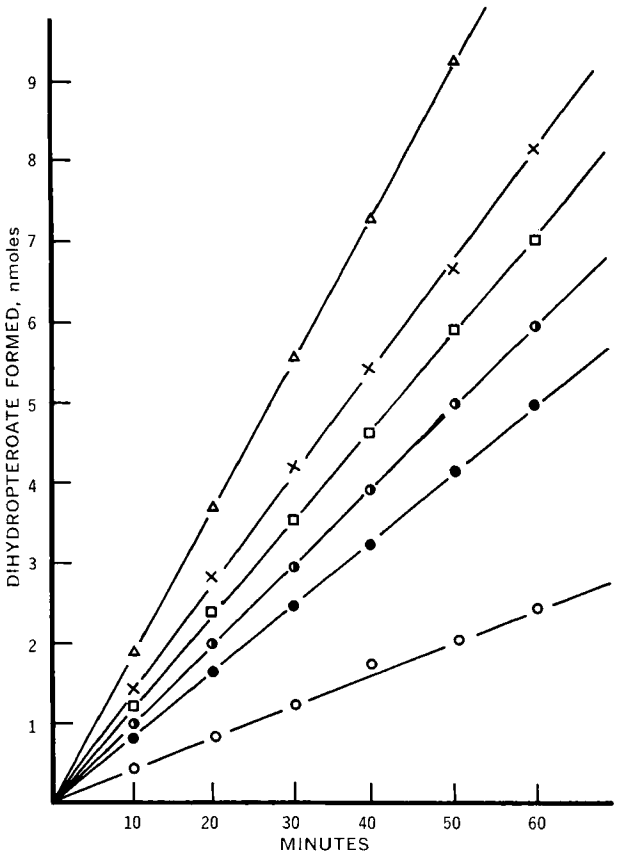

Figure 1—*Enzymic formation of dihydropteroate at 37° using different concentrations of substrate and incubation periods. The total volume of the incubation mixtures was 0.36 ml and included $MgCl_2$, 0.278 mM: mercaptoethanol, 8 mM; tromethamine–hydrochloric acid buffer (pH 8.0), 10 mM; dihydropteroate synthetase, 0.45 mg; and p-aminobenzoic acid-^{14}C, 4.4 × 10^{-2} mM. Key (hydroxymethyldihydropteridine pyrophosphate): ○, 0.034 mM; ●, 0.069 mM; ◑, 0.083 mM; □, 0.11 mM; ×, 0.138 mM; and △, 0.26 mM.*

An attempt was made to obtain the free acid form of the hydroxymethylpteridine pyrophosphate by passing the ammonium salt through a cation exchanger[2]. The free acid form was eluted rapidly and gave a single sharp peak. However, when the pooled fractions were lyophilized, the free acid was found to be less stable than the ammonium salt and it soon polymerized.

The hydroxymethylpteridine pyrophosphate obtained was iden-

[1] Sephadex G-10.
[2] AG 50W-X12.

Table I—Determination of Optimal Concentration of 2-Amino-4-hydroxy-6-hydroxymethylpteridine Pyrophosphate as Substrate for Dihydropteroate Synthetase

Concentration, m*M*	Activity[a], %
0.034	22
0.069	42
0.138	75
0.26	100
0.52	98
1.0	94
1.3	91
2.6	76

[a] Measured with enzyme extracted from *N. meningitidis* strain M-60 EUR, whose minimal inhibitory concentration by sulfanilamide on plates is 1 μg/plate. The extraction procedure was described previously (13).

tical to an authentic sample of this compound[3] in regard to both UV and IR absorption spectra and chromatographic behavior. The IR spectrum showed the presence of primary amino, phosphate hydroxyl, and pteridine ring stretching frequencies, and the proton NMR spectrum showed two methylene protons as a multiplet from splitting by phosphate. Neither IR nor proton NMR spectra nor a melting point have been previously reported for this compound.

Biological Evaluation—Reduction of the hydroxymethylpteridine pyrophosphate to its dihydro form using sodium dithionite (8) gave a product that showed full enzymatic activity with the dihydropteroate synthetase extracted from both *Neisseria meningitidis* and *N. gonorrhoeae*. By using the enzyme from one strain[4] of *N. meningitidis*, rates of formation of dihydropteroate for different concentrations of the hydroxymethyldihydropteridine pyrophosphate were measured.

The dihydropteroate, formed using *p*-aminobenzoate-^{14}C, was eluted from the origin and identified by its characteristic UV spectrum. Total dihydropteroate was radioassayed; this procedure was found to be highly reproducible. The rate of dihydropteroate synthesis was dependent on hydroxymethyldihydropteridine pyrophosphate concentration in the presence of a constant concentration of *p*-aminobenzoate (Fig. 1). A Lineweaver–Burke plot (of the reciprocal of rate of dihydropteroate synthesis *versus* the reciprocal of substrate concentration) gave a K_m value for the hydroxymethyldihydropteridine pyrophosphate of 2×10^{-4} *M* (Fig. 2), obtained from the average of three duplicate experiments. A K_m value was determined by Shiota *et al.* (5), using a cell-free extract from *Veillonella* strain V2 with the pyrophosphate as substrate; their value was 3.12×10^{-4} *M*.

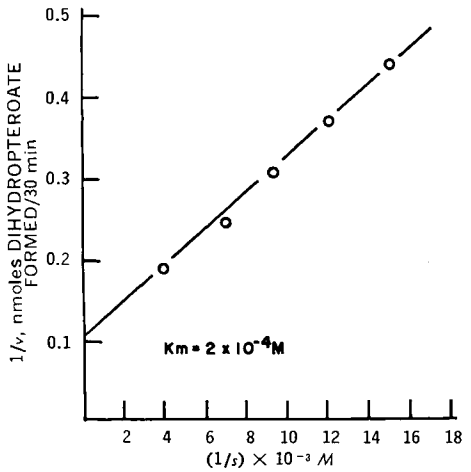

Figure 2—*Effect of hydroxymethyldihydropteridine pyrophosphate concentration on rate of enzymic synthesis of dihydropteroate. Velocity is given in nanomoles of product formed per milligram of protein per 30 min. Concentration of substrate was varied between 6.9 × 10⁻⁵ and 26 × 10⁻⁵ M. Experimental conditions were as described for Fig. 1.*

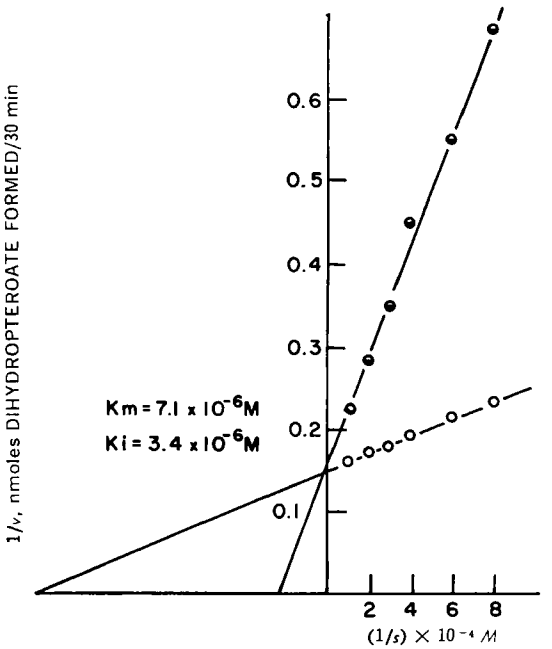

Figure 3—*Inhibition of dihydropteroate synthetase by dapsone (1.8 × 10⁻⁵ M) using varying amounts of p-aminobenzoic acid (0.012–0.098 mM) and a constant amount of hydroxymethylpteridine pyrophosphate (0.26 mM). Key: ○, control; and ◒, with dapsone.*

As shown in Table I, the optimal substrate concentration of the pyrophosphate for the dihydropteroate synthetase employed was 0.26 m*M*. The pyrophosphate becomes inhibitory beyond that concentration.

Inhibition Studies—Inhibition studies using the hydroxymethylpteridine pyrophosphate as substrate for the dihydropteroate synthetase of *N. meningitidis* were carried out using sulfanilamide, sulfadiazine, dapsone, and bis(4-aminophenyl) disulfide. Dapsone competitively inhibited the synthesis of dihydropteroate (Fig. 3), which is in accord with a previous finding that the antibacterial action of dapsone is reversed by *p*-aminobenzoate (9). This has been further substantiated by recent reports that dapsone (10) and the sulfonamides (11) were competitively inhibitory to the same enzyme from a cell-free extract of *Escherichia coli*. The other compounds observed also produced competitive inhibitions at concentrations of 1.7×10^{-5} *M*, in the following order of decreasing activity: sulfadiazine — dapsone > sulfanilamide > bis(4-aminophenyl) disulfide. The disulfide has been postulated to function analogously to dapsone in malaria chemotherapy (12).

EXPERIMENTAL[5]

2-Amino-4-hydroxy-6-hydroxymethylpteridine Pyrophosphate—Pyrophosphoric acid was crushed and dried under vacuum for 4 days, and 25 g was melted slowly in a glass-stoppered flask at 60–65°. 2-Amino-4-hydroxy-6-hydroxymethylpteridine (7) (300 mg, 0.0008 mole) was added slowly, and the flask was protected from light by aluminum foil. The mixture was stirred and heated at 60–65° for 2 hr. After addition of 80 ml of distilled water, the contents were transferred to a beaker, and an aqueous charcoal suspension[6] (3 g in 20 ml) was added. The mixture was stirred for

[3] Kindly supplied by Dr. T. Shiota.

[4] Strain M-60 EUR; obtained from the Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C., through the courtesy of Dr. M. Artenstein.

[5] The melting point was taken on a Mel-Temp apparatus and is uncorrected. Microanalyses were done by Galbraith Laboratories, Knoxville, Tenn. UV absorption spectra were determined with a Cary model 14 spectrophotometer, IR absorption spectra were obtained with a Perkin-Elmer model 137B spectrophotometer, and proton NMR spectra were obtained with a Varian T60 spectrometer using D_2O as the solvent.

[6] Darco 60.

30 min and was filtered through a 0.45-μm filter[7]. The charcoal pad was carefully removed from the filter and washed with 500 ml of distilled water to remove excess pyrophosphoric acid. The pteridine adsorbed on the charcoal pad was eluted by suspending the pad in 75 ml of 3 *N* ammonium hydroxide, and the mixture was stirred for 15 min. The resulting fine suspension was filtered through a fresh filter paper[7]. This procedure was repeated three times with the same quantity of 3 *N* ammonium hydroxide as eluant. The combined filtrate was evaporated under reduced pressure at room temperature until no odor of ammonia could be detected. The solution was lyophilized to yield about 300 mg (80%) of greenish-yellow amorphous powder. An analytical sample was obtained by recrystallization from aqueous ethanol, mp 166–175°; UV λ_{max} (0.1 *N* NaOH): 255 and 362 nm; IR ν_{max} (mineral oil): 3300, 1675 (NH_2), 1240 (P=O), 1040 (P—OH), and 820 (pteridine) cm^{-1}; NMR: δ 5.2 (m, 2H, CH_2) and 7.0 (d, 1H, aromatic H); R_f (water) 0.98.

Anal.—Calc. for $C_7H_{15}N_7O_8P_2$: C, 21.71; H, 3.90; N, 25.30; P, 15.99. Found: C, 21.86; H, 4.10; N, 24.50; P, 15.95.

[7] Millipore.

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Potential Anticancer Agents I: Confirming Evidence for the Structure of Fagaronine

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Abstract □ The structure of fagaronine was confirmed by spectral studies on its *N*-demethyl derivative.

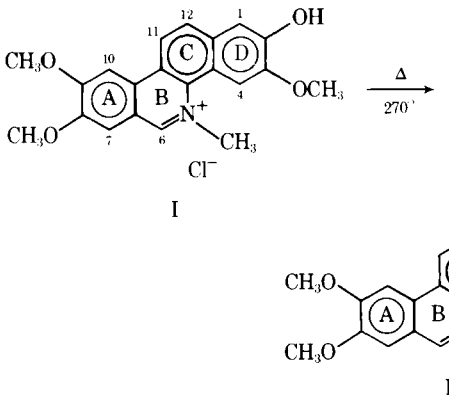
Keyphrases □ Fagaronine—structure confirmation □ Anticancer agents, potential—confirmation of fagaronine structure □ *Fagara zanthoxyloides* (Rutaceae) alkaloids—confirmation of fagaronine structure

Fagaronine (I), an alkaloid of *Fagara zanthoxyloides* (Rutaceae) (1), exhibits potent antileukemic properties and is currently being considered for preclinical evaluation by the National Cancer Institute. In an initial report, two possible structures for fagaronine were suggested, one of which was favored on the basis of mass spectral evidence. At that time, it was not possible to study the NMR spectrum in detail due to the poor solubility of I in all of the usual solvents. This problem has now been overcome by the preparation of *N*-demethylfagaronine (II) (Scheme I) which was found to be soluble in $CDCl_3$ and dimethyl sulfoxide-d_6 and from which good NMR data could be obtained.

DISCUSSION

The NMR spectrum (Table I) of *N*-demethylfagaronine in $CDCl_3$ and that of its hydrogen-bonded complex with triethylamine showed a significant increase in shielding of an upfield aromatic proton (δ7.47) by 0.17 ppm and little or no effect on the other aromatic protons. This increase in shielding would be expected for protons *ortho* and *para* to the phenolic OH position (2). The proton at C-4, which could be expected to produce a downfield signal due to its orientation with respect to the ring B nitrogen and the accompanying electron pair, was not affected, which suggested that the phenolic group was located at the C-2 position.

The use of dimethyl sulfoxide-d_6 as the NMR solvent offered the advantage of determining in the same solvent the chemical shifts of the free *N*-demethyl base as well as those of the phenolate anion of the base. From the chemical shift changes, it was possible



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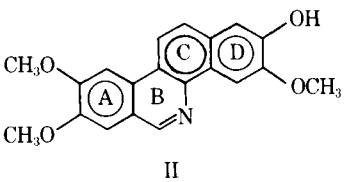

II

Scheme I—Preparation of N-Demethylfagaronine from Fagaronine