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Pyrimidodiazepine, a Ring-Strained Cofactor for Phenylalanine Hydroxylase†

David C. Pike, Mary T. Hora, Steven W. Bailey, and June E. Ayling*
Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, Alabama 36688
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ABSTRACT: Homologues of 6-methyl-7,8-dihydropterin (6-Me-7,8-PH_2) and 6-methyl-5,6,7,8-tetrahydropterin (6-Me-PH_4), expanded in the pyrazine ring, were synthesized to determine the effect of increased strain on the chemical and enzymatic properties of the pyrimidodiazepine series. 2-Amino-4-keto-6-methyl-7,8-dihydro-3*H*,9*H*-pyrimido[4,5-*b*][1,4]diazepine (6-Me-7,8-PDH_2) was found to be more unstable in neutral solution than 6-Me-7,8-PH_2. Its decomposition appears to proceed by hydrolytic ring opening of the 5,6-imine bond, followed by autooxidation. 6-Me-7,8-PDH_2 can be reduced, either chemically or by dihydrofolate reductase (K_m = 0.16 mM), to the 5,6,7,8-tetrahydro form (6-Me-PDH_4). This can be oxidized with halogen to quinoid dihydropyrimidodiazepine (quinoid 6-Me-PDH_2), which is a substrate for dihydropteridine reductase (K_m = 33 μM). Whereas quinoid 6-methyldihydropterin was found to tautomerize to 6-Me-7,8-PH_2 in 95% yield in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, quinoid 6-Me-PDH_2 gives only 53% 6-Me-7,8-PDH_2, the remainder decomposing via an initial opening of the diazepine ring. Additional evidence for the extra strain in the pyrimidodiazepine system is the cyclization of quinoid 6-*N*-(2′-aminopropyl)divicine to quinoid 6-Me-PH_2 in 57% yield in 0.1 M Tris-HCl, pH 7.4. By comparison, no quinoid 6-Me-PDH_2 is formed from the homologue quinoid 6-*N*-(3′-aminobutyl)divicine. A small (2%) yield of 6-Me-PDH_4 is found if the unstable C4a-carbinolamine intermediate is trapped by enzymatic dehydration and reduction. Although phenylalanine hydroxylase utilizes 6-Me-PDH_4 (K_m = 0.15 mM), the maximum velocity of tyrosine production is 20 times slower than that with 6-Me-PH_4, indicating that a ring opening reaction is not a rate-limiting step in the hydroxylase pathway. Further, the maximum velocities of 2,5,6-triamino-4(3*H*)-pyrimidinone, 2,6-diamino-5-(methylamino)-4(3*H*)-pyrimidinone, and 2,6-diamino-5-(benzylamino)-4(3*H*)-pyrimidinone span a 35-fold range. These cofactors would theoretically form the same oxide of quinoid divicine if oxygen activation involves a carbonyl oxide intermediate. Thus, the limiting step is also not transfer of oxygen from this hypothetical intermediate to the phenylalanine substrate.

Phenylalanine hydroxylase is a tetrahydrobiopterin-dependent aromatic amino acid monooxygenase that converts excess dietary phenylalanine to *p*-tyrosine. Isotopic labeling of pyrimidine cofactor analogues (Bailey et al., 1982) and detection of a C4a-hydroxypterin product (Kaufman, 1975; Lazarus et al., 1982) have demonstrated that activation of molecular oxygen in this reaction involves its covalent addition to cofactor. The initial adduct has not yet been observed. A C4a-hydroperoxide of the structurally related dihydroflavin has been shown to be an early intermediate of several enzymes (Ballou, 1984). Hamilton has proposed that a highly reactive carbonyl oxide can be generated from the C4a-hydroperoxide of either cofactor by cleavage of their respective C4a–N5 bonds (Hamilton, 1974). The direct utilization of the hydroperoxide vs. this ring-opened mechanism is outlined in Figure 1. Although model studies have shown that flavin C4a-hydroperoxides may be sufficiently potent to account in themselves for the oxidation of many flavoprotein substrates (Bruice, 1984), the possibility of ring-opened intermediates, especially of *p*-hydroxybenzoate and phenol hydroxylases, is still debated (Detmer & Massey, 1985). Beyond the involvement of position C4a of cofactor, little is known of the structure of the ultimate oxygen reagent formed by phenylalanine hydroxylase; for example, no evidence yet affirms or refutes a pyrazine ring cleavage mechanism.

The effect of ring size on inherent strain and ring opening and closure reactions has been studied in many systems. In most cases, seven-membered rings are more strained than their six-membered counterparts. A homologue of 6-methyltetra-

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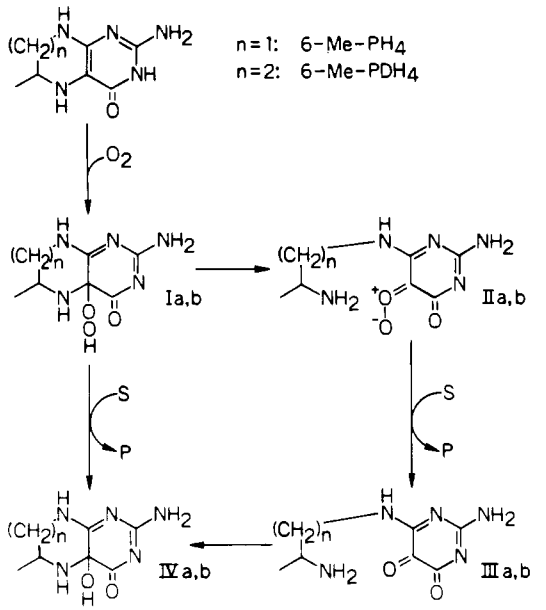

FIGURE 1: Comparison of direct utilization of a cofactor C4a-hydroperoxide with the ring-opened carbonyl oxide as possible pathways of oxygen activation by phenylalanine hydroxylase: (a) (n = 1) 6-methyltetrahydropterin and (b) (n = 2) the homologue 2-amino-4-keto-6-methyltetrahydropyrimidodiazepine.

hydropterin (6-Me-PH_4),[1] 2-amino-4-keto-6-methyl-5,6,7,8-tetrahydro-3H,9H-pyrimido[4,5-b][1,4]diazepine (6-Me-PDH_4), was synthesized to observe the chemical and enzymatic properties of pyrazine ring expansion. The extra methylene group was found to significantly increase the instability of the 7,8-dihydro and quinoid dihydro forms of the new analogue toward ring opening reactions. A further indication of additional strain in the pyrimidodiazepine series was the reduced yield of quinoid 6-Me-PDH_2 in the spontaneous cyclization of IIIb (Figure 1). If, during the phenylalanine hydroxylase reaction ring cleavage occurs, and is rate-limiting, then the extra strain in a pyrimidodiazepine cofactor should result in a faster reaction than with the analogous pterin.

The transfer of oxygen from the proposed carbonyl oxide to phenylalanine was probed by examination of the velocity of the hydroxylase with 5-substituted amino derivatives of 2,5,6-triamino-4(3H)-pyrimidinone (TP) as cofactors (V, Figure 2) (Bailey & Ayling, 1978). If the observed loss of the 5-amino group from these compounds is a result of Hamilton's proposed mechanism, a common intermediate, the carbonyl oxide of quinoid divicine (VII), results regardless of any substitution of that amine (Figure 2). A detailed comparison of TP and two 5-amino derivatives was undertaken to determine for each the absolute maximum velocity of phenylalanine hydroxylation. If utilization of the hypothetical

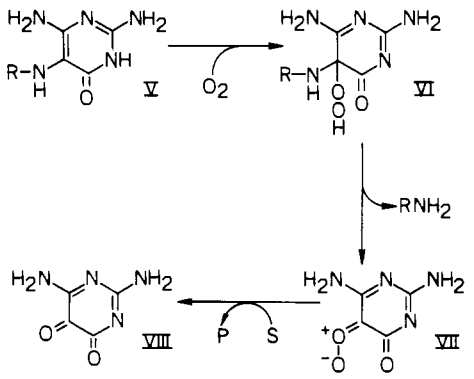

FIGURE 2: Hypothesized carbonyl oxide pathway as applied to 5-N derivatives of 2,5,6-triamino-4(3H)-pyrimidinone (V).

oxene reagent is rate-limiting, the maximum rate of hydroxylation should not be affected by the 5-amino substituent.

Materials and Methods

Enzymes. Dihydropteridine reductase (DHPR) from bovine liver was purified and assayed as previously described (Bailey & Ayling, 1983). Phenylalanine hydroxylase from rat liver was purified by the method of Shiman et al. (1979). A unit of phenylalanine hydroxylase is defined as 1 μmol of tyrosine formed per minute in the presence of 0.2 mM 6-methyltetrahydropterin (6-Me-PH_4), 1.0 mM phenylalanine, atmospheric oxygen, and 0.1 M pH 7.4 Tris-HCl, at 27 °C. Phenylalanine hydroxylase stimulating protein (PHS) was purified 200-fold from rat livers by the method of Huang et al. (1973), but omitting the phosphocellulose column. Bovine liver dihydrofolate reductase (DHFR), horseradish peroxidase type II, and bovine erythrocyte superoxide dismutase were purchased from Sigma. Bovine liver catalase was from Worthington.

Reagents. 6-Methyltetrahydropterin was obtained from Dr. B. Schircks, Jona, Switzerland, 4-chloro-2-butanone from Pfaltz and Bauer, 1,3-dibromobutane from Aldrich, and 1,2-diaminopropane from Fluka. 2,6-Diamino-5-(methylamino)-4(3H)-pyrimidinone (5N-Me-TP) was prepared by the procedure of Pfleiderer and Sagi (1964) and Haines et al. (1962). 2,5,6-Triamino-4(3H)-pyrimidinone dihydrochloride and 2,6-diamino-5-(benzylamino)-4(3H)-pyrimidinone (5N-Bz-TP) were prepared as previously published (Bailey & Ayling, 1978).

Analytical Procedures. Specific chromatographic procedures are given in the text where utilized. Many of these, especially for separation of the pyrimidine derivatives, required a guard column packed with a stationary phase matching that in the main column, since inappropriate retentions on pellicular materials were observed. A dual glassy carbon electrode, BAS MF1000 plus MF1018, was used for electrochemical detection. Mass spectra were acquired on a Hewlett-Packard Model 5982A spectrometer equipped with a 5933 data system. Tyrosine was analyzed by HPLC with fluorescence detection (Bailey & Ayling, 1980a).

Measurement of K_m Values for Oxygen. The rate of phenylalanine hydroxylase at various oxygen concentrations was measured in a variable volume (100–300 μL) reaction chamber machined in a Plexiglas block. One wall of the chamber was a YSI Model 5331 Clark oxygen probe. A glass-encapsulated micro magnetic stir bar was spun on the horizontal axis common to the oxygen probe. The chamber was open to the atmosphere via a vertical column. Each reaction was maintained at fixed oxygen tensions by oxygen/argon mixtures, set at nominal ratios by calibrated flow gauges, introduced just above the liquid level, and which continuously swept the

[1] Abbreviations: 6-Me-PDH_4, 2-amino-4-keto-6-methyl-5,6,7,8-tetrahydro-3H,9H-pyrimido[4,5-b][1,4]diazepine; TP, 2,5,6-triamino-4(3H)-pyrimidinone; DHPR, dihydropteridine reductase; DHFR, dihydrofolate reductase; 6-Me-PH_4, 6-methyl-5,6,7,8-tetrahydropterin; pterin, 2-amino-4(3H)-pteridinone; 5N-Me-TP, 2,6-diamino-5-(methylamino)-4(3H)-pyrimidinone; 5N-Bz-TP, 2,6-diamino-5-(benzylamino)-4(3H)-pyrimidinone; HPLC, high-pressure liquid chromatography; 6-Me-7,8-PDH_2, 2-amino-4-keto-6-methyl-7,8-dihydro-3H,9H-pyrimido[4,5-b][1,4]diazepine; divicine, 2,6-diamino-5-hydroxy-4(3H)-pyrimidinone; quinoid 6-Me-PH_2, quinoid 6-methyldihydropterin; 6-Me-7,8-PH_2, 6-methyl-7,8-dihydropterin; 6-D-6-Me-PH_2, 6-deuterio-6-methyldihydropterin; quinoid 6-Me-PDH_2, quinoid 2-amino-4-keto-6-methyldihydropyrimido[4,5-b][1,4]diazepine; Na_2EDTA, ethylenediaminetetraacetic acid disodium salt; PHS, phenylalanine hydroxylase stimulating protein (a protein with 4a-carbinolamine dehydratase activity); 6-Me-P, 6-methylpterin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

vertical column. Several additional bores surrounding the main chamber were used to thermostat the reaction with a circulating water bath. This arrangement allowed multiple samples to be withdrawn, with a positive-displacement pipet, as a function of time without disturbing the oxygen concentration. The response of the Clark electrode, conditioned by a current amplifier constructed around an Analog Devices 235J Op Amp, was monitored continuously on a strip chart recorder.

Enzyme Kinetic Constants. Apparent K_m's for phenylalanine hydroxylase were measured with the nonvariable substrate concentrations as follows: cofactor, 0.2 mM; phenylalanine, 1 mM; atmospheric oxygen. The K_m's for cofactor and oxygen have been shown to be independent of the concentration of phenylalanine and vice versa (Kaufman & Fisher, 1974). Maximum velocities were therefore calculated by correction to infinite phenylalanine concentration. This correction was applied to the O_2 K_m curves for the four cofactors that have high O_2 K_m's (6-Me-PDH_4, TP, 5-N-Bz-TP, 5-N-Me-TP) and to the cofactor K_m curve with 6-MePH_4, which has a relatively low K_m for O_2. The concentration of oxygen and K_m for cofactor have been shown to be inversely related (Kaufman & Fisher, 1974). The range of maximum velocities (given in Tables I and II) reflect the possible influence of oxygen concentration on cofactor K_m. Maximum velocities and Michaelis constants were calculated by a nonlinear regression program (Schreiner et al., 1985).

SYNTHESIS

A natural pyrimidodiazepine has been shown to be a precursor to the drosopterins, the red eye pigments in *Drosophila melanogaster* (Wiederrecht et al., 1981; Jacobson et al., 1982). Although some pyrimido[4,5-*b*][1,4]diazepines have been synthesized [e.g., Israel et al. (1970) and Senda et al. (1977)], previous efforts have not been directed toward preparation of homologues of dihydro- or tetrahydropterins. The following method is developed from that used by Stuart and Wood (1963) for synthesis of xanthopterin and by Pfleiderer and Zondler (1966) for 6-Me-7,8-PH_2, in which a chloronitropyrimidine is condensed with a latent or protected aminoketone.

Synthesis of 6-Methyltetrahydropyrimidodiazepine (Figure 3)

N-(3′-Ketobutyl)phthalimide (IX). A suspension of 4.63 g (25.0 mmol) of potassium phthalimide and 2.66 g (25.0 mmol) of 4-chloro-2-butanone in 50 mL of dimethylformamide was heated with stirring at a bath temperature of 90 °C for 21 h. The mixture was then cooled to room temperature and filtered free of solid (KCl), and the solvent was removed under reduced pressure. Remaining dimethylformamide was removed by repeated evaporation of added ethanol. Most of the yellow coloration in the residue was removed by stirring with 2.5 mL of cold ethanol, followed by filtration and drying under vacuum, yielding 5.1 g of pale yellow solid. Crystallization from 25 mL of absolute ethanol yielded 4.64 g (21.4 mmol, 85%) (4.46 g first crop, 0.18 g second crop) of *N*-(3′-ketobutyl)phthalimide as a very pale yellow crystalline solid [mp 110–112.5 °C, lit. mp 112–113 °C (Macholan, 1969)].

N-[3′-(Hydroxyimino)butyl]phthalimide (X). A solution of 4.34 g (20.0 mmol) of IX and 2.08 g (30.0 mmol) of hydroxylamine hydrochloride in 18 mL of pyridine and 20 mL of absolute ethanol was stirred at room temperature for 15 min. The reaction was monitored by HPLC: Zorbax ODS (10 × 0.46 cm); 65% ammonium acetate (20 mM in ammonia), pH 4.8/35% methanol, 1.5 mL/min; UV absorbance at 295 nm. The solution was cooled at −20 °C for 2 h; the

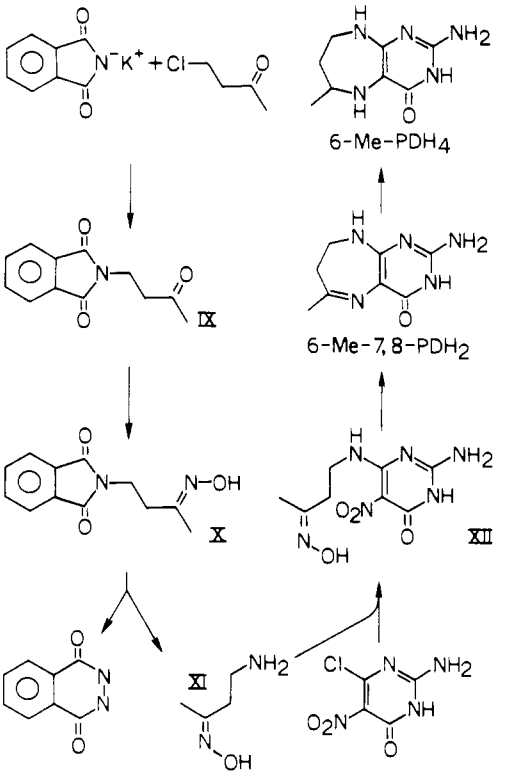

FIGURE 3: Synthesis of 7,8-dihydro- and 5,6,7,8-tetrahydro-2-amino-4-keto-6-methylpyrimidodiazepines (6-Me-7,8-PDH_2 and 6-Me-PDH_4, respectively).

precipitate filtered, washed with cold ethanol, and dried under reduced pressure. Further product was recovered from the filtrate by removal of solvent under reduced pressure; the solid was stirred with 5 mL of cold ethanol, filtered, and dried. The combined product was crystallized from 110 mL of absolute ethanol, yielding 4.46 g (19.2 mmol, 96%) of *N*-[3′-(hydroxyimino)butyl]phthalimide as a white crystalline solid.

1-Amino-3-butanone Oxime Hydrochloride (XI). To a solution of 1.0 g (4.3 mmol) of X in 80 mL of absolute ethanol in an 80 °C bath was added with stirring in one portion 360 mL of anhydrous hydrazine (97%) (11.36 mmol, 2.6 equiv). Phthalhydrazide started precipitating after 1.5 h, and by 3 h all phthalimide starting material had been consumed. The reaction was monitored by HPLC: Zorbax ODS (10 × 0.46 cm); ammonium formate (20 mM in ammonia), pH 3.3, with linear methanol gradient from 12 to 60% in 12 min, 1.5 mL/min; UV absorbance at 300 nm. The mixture was then cooled in an ice bath and acidified with 1 mL of 6 N HCl to pH 4.0. This was stored at −15 °C overnight, the precipitate removed by centrifugation, and the supernatant evaporated under reduced pressure to a pale yellow solid. This was mixed with 6 mL of water, most of the remaining phthalhydrazide removed by centrifugation, and the supernatant evaporated to 830 mg of pale yellow hygroscopic solid. Product was analyzed for primary amines as their *o*-phthalaldehyde/2-mercaptoethanol derivatives (Lindroth & Mopper, 1979) by HPLC: Spherisorb ODS 1 (25 × 0.46 cm); 55% ammonium phosphate (50 mM in ammonia), pH 6.5/45% methanol, 1.5 mL/min; fluorescence detection 325-nm excitation and 460-nm emission. The 1-amino-3-butanone oxime hydrochloride (XI) was purified by fractional crystallization twice from absolute ethanol, with hydrazine hydrochloride crystallizing first as long needles, resulting in 365 mg (61% yield) of colorless crystals free of any other *o*-phthalaldehyde positive impurities.

2-Amino-6-[[3′-(hydroxyimino)butyl]amino]-5-nitro-4-(3H)-pyrimidinone (XII). A solution of 2-amino-6-chloro-

5-nitro-4(3*H*)-pyrimidinone was made by dissolving 427 mg (2.05 mmol) of freshly prepared hydrate (Bailey & Ayling, 1983) in 90 mL of boiling absolute ethanol. The solution was filtered while hot to remove 19 mg of insoluble impurity. The filtrate was heated in an 80 °C bath, and 277 mg (2.0 mmol) of solid hydrochloride (XI) was added. The progress of the reaction was monitored by HPLC: Partisil SCX (25 × 0.46 cm); ammonium acetate (20 mM in ammonia), pH 4.8, 1.5 mL/min; UV absorbance at 198 and 335 nm. Triethylamine was added in aliquots, total 700 μL (5.0 mmol), until it failed to cause any further decrease in chloropyrimidine. After a total of 8 h, the reaction mixture was cooled in an ice bath and 456 mg of fine pale yellow solid removed by centrifugation and dried under vacuum. A second crop of 25 mg was collected for a total yield of 94%. Mass spectrum (EI, 70 eV) showed the following: *m/z* (% rel abundance) 256 (M^+, 11), 239 (24), 221 (14), 184 (100), 180 (25), 164 (18), 155 (27), 138 (32), 126 (29), and 111 (38).

2-Amino-4-keto-6-methyl-7,8-dihydro-3H,9H-pyrimido[4,5-b][1,4]diazepine (6-Me-7,8-PDH$_2$). A suspension of 128 mg of XII in 5.5 mL of dimethylformamide, 0.25 mL of 1 N NaOH, and 5.25 mL of H_2O was thoroughly argonated and warmed in a 60 °C bath. Solid sodium dithionite, totaling 368 mg (2.1 mmol), was added in six portions until HPLC analysis [Partisil SCX (25 × 0.46 cm); 80% ammonium formate (0.1 M in ammonia), pH 3.3/20% methanol, 1.5 mL/min; UV absorbance at 335 nm] indicated that greater than 98% of XII had been consumed. After cooling in an ice bath and centrifugation to remove yellow insoluble material that had been present in the nitropyrimidine, 1 M aqueous $BaCl_2$ was added in aliquots, followed by centrifugation, until no more precipitate formed. The supernatant was evaporated to dryness under reduced pressure, and product was extracted from the residue with 1-mL portions of thoroughly argonated methanol. The combined methanolic extracts were quickly acidified with 0.5 mL of 6 N HCl and evaporated to 333 mg of pale yellow solid. Chromatographic analysis showed that 92% of the absorbance of this material at 326 nm resided in the peak due to 6-Me-7,8-PDH$_2$. A yield of 0.32 mmol (60% from XI, the balance of the weight being salt) was estimated by UV absorbance at 326 nm. The extinction coefficient of 6-Me-7,8-PDH$_2$ was determined by quantitative reduction to 6-Me-PDH$_4$ either by $NaBH_4$ or dihydrofolate reductase/NADPH. UV (0.1 N HCl) showed the following: λ_{max} (ϵ) 215 (16500), 250 (12700), 267 (9200), and 325 (8800). Mass spectrum (EI) showed the following: *m/z* (% rel abundance) 193 (M^+, 100), 192 (25), 178 (14), 165 (15), and 152 (18). For stability studies and determination of enzyme substrate parameters, 6-Me-7,8-PDH$_2$ was purified by preparative HPLC: R-Sil SCX (25 × 0.92 cm); 65% ammonium formate (0.1 M in ammonia), pH 3.3/35% methanol, 6 mL/min.

2-Amino-4-keto-6-methyl-5,6,7,8-tetrahydro-3H,9H-pyrimido[4,5-b][1,4]diazepine (6-Me-PDH$_4$). To an argonated solution of 312 mg of crude 6-Me-7,8-PDH$_2$ (0.30 mmol, see above) at 23 °C was added $NaBH_4$ (5 M in water) in 30-μL portions until HPLC analysis [Partisil SCX (25 × 0.46 cm); 90% ammonium formate (0.1 M in ammonia), pH 3.3/10% methanol, 2.0 mL/min; UV absorbance at 271 nm] indicated greater than 98% conversion to 6-Me-PDH$_4$ (0.75 mmol of $NaBH_4$ total). The mixture was acidified with 200 μL of 6 N HCl and evaporated under reduced pressure to a pale yellow solid. This material was purified by preparative HPLC: R-Sil SCX (25 × 0.92 cm); 85% ammonium formate (0.1 M in ammonia), pH 3.3/15% methanol, 6.0 mL/min. The extinction coefficient was determined by titration with dichlorophenolindophenol. UV (0.1 N HCl) showed the following: λ_{max} (ϵ) 215 (16000) and 271 (14900). Mass spectrum (EI) showed the following: *m/z* (% rel abundance) 195 (M^+, 56), 180 (100), and 152 (27).

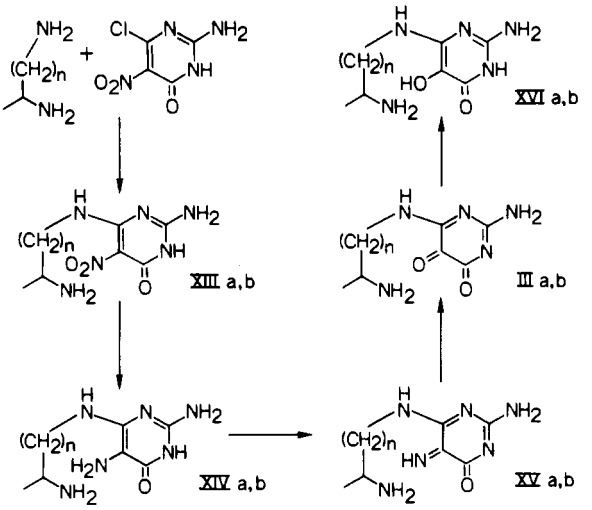

FIGURE 4: Synthesis of quinoid 6-*N*-(2′-aminopropyl)- and 6-*N*-(3′-aminobutyl)divicines (IIIa and IIIb): (a) $n = 1$; (b) $n = 2$.

Synthesis of N6 Derivatives of TP (XIVa,b) and Divicine (XVIb) (Figure 4)

2-Amino-6-[(3′-aminobutyl)amino]-5-nitro-4(3H)-pyrimidinone Hydrochloride (XIIIb). To a solution of 1.04 g (5.0 mmol) of 2-amino-6-chloro-5-nitro-4(3*H*)-pyrimidinone hydrate in 275 mL of absolute ethanol at reflux was added 0.44 g (5.0 mmol) of 1,3-diaminobutane, prepared from 1,3-dibromobutane (Campbell & Urbach, 1973). Precipitation of product began shortly after addition of the diamine. The progress of the reaction was monitored by HPLC: Partisil SCX (10 × 0.46 cm); 80% ammonium acetate (0.2 M in ammonia), pH 4.8/20% methanol, 2.0 mL/min; UV absorbance at 335 nm. After 1.5 h the mixture was allowed to cool. The precipitate was collected by centrifugation and the supernatant concentrated to 8 mL and stored at −15 °C overnight to obtain a second crop. A small contamination with the 3′-amino-1′-methylpropyl isomer was removed from the combined product (0.96 g) by extraction into a total of 425 mL of 5 mM HCl in methanol by sonication. After the residue was removed by centrifugation, the solution was concentrated to 200 mL and the product precipitated by addition of 350 mL of diethyl ether and dried: 0.54 g (39% yield) of white solid.

2,5-Diamino-6-[(3′-aminobutyl)amino]-4(3H)-pyrimidinone Dihydrochloride [6-N-(3′-Aminobutyl)-TP] (XIVb). A solution of XIIIb, 0.28 g (1.0 mmol), in 10 mL of dimethylformamide, 10 mL of H_2O, and 0.25 mL of 1 M NaOH was thoroughly argonated and heated to 60 °C. Sodium dithionite was added, in 0.17-g (1.0-mmol) portions, with vigorous stirring in quick succession until a nearly colorless solution was produced (0.85 g, 5 mmol total). After this was cooled on ice, 1 M $BaCl_2$ was added until precipitation ceased (2.5 mL total). After centrifugation, the supernate together with 0.35 mL of HCl (concentrated) was evaporated to dryness under reduced pressure. The residue was extracted under argon with methanol and product precipitated by addition of 3 volumes of diethyl ether. An approximate yield (64%) was determined by an absorption spectrum using an extinction coefficient of 16700 at λ_{max} = 269 nm in 0.1 N HCl [determined by dichlorophenolindophenol titration and correcting for the area percent residing in the product peak (92%) when analyzed by

HPLC: Partisil SCX (25 × 0.46 cm); 80% ammonium acetate (0.2 M in ammonia), pH 4.8/20% methanol, 2 mL/min].

2-Amino-6-[(3′-aminobutyl)amino]-5-hydroxy-4(3H)-pyrimidinone [*6-N-(3′-Aminobutyl)divicine*] (*XVIb*). All of the 6-N-(3′-aminobutyl)-TP (XIVb) resulting from the above procedure (approximately 0.64 mmol plus salt) was dissolved in a minimal volume of well-argonated methanol (16 mL) and cooled in an ice bath. Bromine, 50 μL (0.95 mmol), was added with stirring. Hydrolysis of the resulting quinoid TP derivative (XVb) by the water present in the solvent to give quinoid 6-*N*-(3′-aminobutyl)divicine (IIIb) was complete by 10 min. The solution was then quickly evaporated to an oil under reduced pressure, and 4 mL of 1 M 2-mercaptoethanol was added, followed by 114 mg of ammonium carbonate (1.0 mmol), to give a final pH of approximately 4. After a few minutes, the water was removed by evaporation at reduced pressure, and the residue was acidified with 2 mL of 1 M methanolic HCl and dissolved in a minimum volume of additional methanol. Product (XVIb) was then precipitated with 10 volumes of diethyl ether, washed with additional diethyl ether, and dried under vacuum.

2-Amino-6-[(2′-aminopropyl)amino]-5-nitro-4(3H)-pyrimidinone hydrochloride (*XIIIa*) was made by the procedure for XIIIb above using 1,2-diaminopropane. Product was purified by precipitation from methanol/HCl with diethyl ether 3 times in order to remove a small amount of the 6-(1′-aminoisopropyl) derivative.

2,5-Diamino-6-[(2′-aminopropyl)amino]-4(3H)-pyrimidinone [*6-N-(3′-aminopropyl)-TP*] (*XIVa*) was made from XIIIa by the procedure for XIVb.

Results

The effect of the additional methylene group on the strain differential between the pterins and pyrimidodiazepines was determined by measuring the rate of ring opening of their respective 7,8-dihydro and quinoid dihydro forms as well as the opposing cyclization reaction that generates these quinoids.

Cyclization of Quinoid 6-N-(2′-Aminopropyl)divicine (*IIIa*). A 10 mM solution of XIVa in 0.01 N HCl was prepared on the basis of an assumed extinction coefficient of 16 000 M^{-1} cm^{-1} at 269 nm in 0.1 N HCl. A 50-μL aliquot was equilibrated to 27 °C, and 10.5 μL of 50 mM I_2 in methanol (5% excess) added. The XVa thus produced was allowed to hydrolyze to the quinoid divicine (IIIa). The rate of formation of the carbinolamine intermediate (IVa) from IIIa is primarily, although not exclusively, influenced by the charge of the exocyclic amino group and is very slow in the initially acidic medium (S. W. Bailey and J. E. Ayling, unpublished results). After 3 min, 940 μL of 0.1 M Tris-HCl, pH 7.4, at 27 °C was added. Cyclization was monitored chromatographically in two ways.

(a) Samples of the above mixture were injected directly into the chromatograph: R-Sil SCX (25 × 0.40 cm); 85% ammonium formate (0.3 M in ammonia), pH 3.3/15% methanol, 1.5 mL/min. The remaining IIIa, the quinoid dihydropterin, and the 7,8-dihydropterin are detected by their absorbance at 270 nm, and the two quinoid compounds are detected also by electrochemical reduction at −0.1 V with respect to an Ag/AgCl reference. The peak due to quinoid 6-Me-PH_2 represents the sum of this compound plus any C4a-hydroxy pterin that might have been in the reaction, since the latter carbinolamine is instantly dehydrated on first contact with pH 3.3 eluant (S. W. Bailey and J. E. Ayling, unpublished results).

(b) Samples of the neutralized solution of IIIa were also diluted 1:1 into 0.1 M ascorbic acid. The resulting mixture of 6-*N*-(2′-aminopropyl)divicine (XVIa) and 6-Me-PH_4 was cooled on ice and analyzed by HPLC: Partisil SCX (25 × 0.46 cm); ammonium formate (0.1 M in ammonia), pH 3.3, including 1.0 mM Na_2EDTA, 1.5 mL/min; detection by absorbance at 280 nm and by electrochemical oxidation at 0.3 V with respect to Ag/AgCl. As above, 6-Me-PH_4 results from the sum of both the quinoid 6-Me-PH_2 and the C4a-hydroxy pterin present at the time of addition of ascorbate. The initial concentration of IIIa, determined by substitution of 940 μL of distilled water for the Tris-HCl buffer, was analyzed by methods a and/or b.

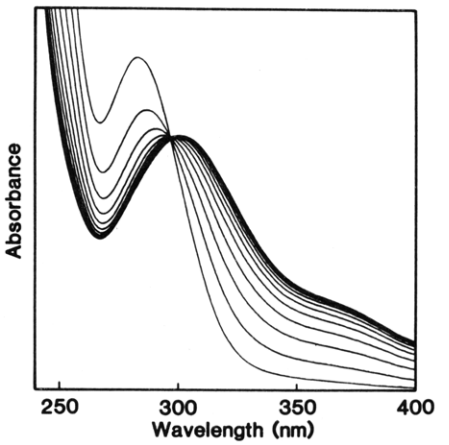

FIGURE 5: Cyclization of quinoid 6-*N*-(2′-aminopropyl)divicine (IIIa) to quinoid 6-Me-PH_2 in 0.1 M Tris-HCl, pH 7.4, at 17 °C; 30 s between scans.

In 0.1 M Tris-HCl at pH 7.4 and 27 °C, quinoid 6-*N*-(2′-aminopropyl)divicine (IIIa) disappears at a rate of 0.099 ± 0.003 s^{-1} ($t_{1/2}$ = 7.0 s), determined primarily by method b and verified by method a. Of the IIIa that is consumed, only 57 ± 4% is found as the combination of either quinoid 6-Me-PH_2 and 6-Me-7,8-PH_2 (method a) or 6-Me-PH_4 and 6-Me-7,8-PH_2 (method b). The remainder of the starting material appears as a number of uncharacterized products that absorb only in the low UV (see below). If all of the nonproductive pathway is attributed to the direct decomposition of IIIa, the rate of the initial attack by the exocyclic amine on the C5 carbonyl of the pyrimidine is approximately 0.056 s^{-1}.

At pH 7.4, and 27 °C, IIIa disappears following first-order kinetics, falling below 0.01% of the original material (the minimum detectable level of method a). Considering the above yield, the ΔG of cyclization must be less than −5.1 kcal M^{-1}. The reverse reaction was investigated with quinoid 6-deuterio-6-methyldihydropterin (quinoid 6-D-6-Me-PH_2), which rearranges 9.8 times more slowly to the 7,8-dihydropterin than the undeuterated material (Armarego et al., 1984). When 6-deuterio-6-methyltetrahydropterin in oxidized to the quinoid dihydropterin by stoichiometric iodine, no IIIa can be detected by method a regardless of the incubation time in 0.1 M Tris-HCl, pH 7.4, at 27 °C.[2]

Repeat spectra of the condensation of IIIa show that at pH 7.4 dehydration of the carbinolamine is limiting (Figure 5). The rate of dehydration at 27 °C of 0.036 s^{-1} can be obtained from the best fit to the progress curve at 340 nm (the isosbestic point between quinoid 6-Me-PH_2 and 6-Me-7,8-PH_2). Taking the pH difference into account, this last figure is consistent with that observed for dehydration of the C4a-hydroxy adduct of quinoid 6-Me-PH_2 generated by phenylalanine hydroxylase

[2] The equilibrium described in a preliminary report of this work was found to be due to inadequate time of incubation during hydrolysis of **7a** (Pike et al., 1984).

at pH 8.5 (Lazarus et al., 1983).

Noncyclization of Quinoid 6-N-(3'-Aminobutyl)divicine (IIIb). A 10 mM solution of XIVb in 0.01 N HCl was oxidized with iodine and allowed to hydrolyze in a manner identical with that of XIVa above. After 3 min the resulting IIIb was diluted 20-fold into 0.1 M Tris-HCl, pH 7.4, at 27 °C. Samples were withdrawn at 15-s intervals and diluted 1:1 with 0.1 M ascorbic acid. The resulting 6-*N*-(3'-aminobutyl)divicine (XVIb) and any 6-Me-PDH_4 were separated on Partisil SCX (25 × 0.4 cm), eluted with 90% ammonium formate (0.2 M in ammonia) pH 3.3, containing 1.0 mM Na_2EDTA/10% methanol, and detected by both absorbance at 270 nm and electrochemical oxidation at 0.3 V vs. Ag/AgCl. Direct analysis of quinoid 6-Me-PDH_2 by HPLC, as in method a above, is hindered by its instability in acidic eluants (see next section of Results).

Cyclization of IIIb, as determined by analysis for 6-Me-PDH_4, could not be detected. The method would have revealed a minimum yield of 0.4%. Instead, IIIb decomposed with a first-order rate constant of 0.043 s^{-1} ($t_{1/2}$ = 16 s).

To determine whether a slow rate of dehydration of any carbinolamine intermediate contributes to the lack of observed cyclization, the dehydratase property of phenylalanine hydroxylase stimulating protein (PHS) (Lazarus et al., 1983) was utilized. Ten aliquots of 0.1 mM XVIb of 5 μL each were added at 1-min intervals to a reaction mixture consisting of, upon final addition, 50 nmol of NADH, 450 units of catalase, 0.1 unit of dihydropteridine reductase (DHPR), 45 units of superoxide dismutase, and 100 units of PHS in a total volume of 200 μL of 0.1 M Tris-HCl, pH 7.4, at 27 °C. Autooxidation of XVIb rapidly generates IIIb. After a further 5-min incubation, protein was precipitated with 20 μL of 6 M trichloroacetic acid and the supernate analyzed for the presence of 6-Me-PDH_4 by the above chromatographic system. The rationale for this experiment depends upon the observation that, in contrast to the action of ascorbic acid, IIIb is an extremely poor substrate for DHPR and is not reduced back to XVIb. Quinoid 6-Me-PDH_2 is, however, a good substrate for the reductase (see below):

$$\text{XVIb} \underset{\text{DHPR}}{\overset{O_2}{\rightleftharpoons}} \text{IIIb} \leftrightarrow$$

$$\text{IVb} \overset{\text{PHS}}{\leftrightarrow} \text{quinoid 6-Me-PDH}_2 \xrightarrow{\text{DHPR/NADH}} \text{6-Me-PDH}_4$$

This reaction produced 0.11 nmol of 6-Me-PDH_4 for a yield of 2.2%, none being observed if PHS is omitted.

When the above procedure is repeated except for the substitution of 0.1 M sodium phosphate, pH 6.5, or 0.1 M Tris-HCl, pH 8.9, as buffer, the yield of 6-Me-PDH_4 is halved and doubled, respectively. This behavior is consistent with the pH dependency of the rate of initial intramolecular condensation of IIIa (S. W. Bailey and J. E. Ayling, unpublished results).

Tautomerization of Quinoid 6-Methyldihydropyrimidodiazepine (Quinoid 6-Me-PDH_2). A solution of quinoid 6-Me-PDH_2 was prepared by addition of 0.05 mL of 1.0 mM 6-Me-PDH_4 to a thoroughly argonated mix of 0.1 mL of 1.0 M Tris-HCl, pH 7.4, at 27 °C and 0.7 mL of water, followed by 0.15 mL of 0.5 mM I_2 in water. The reaction was performed anaerobically to prevent oxidative decomposition of the resulting 6-Me-7,8-PDH_2 (see below). Aliquots were removed at 3-min intervals and diluted 1:1 with fresh 0.1 M $Na_2S_2O_4$ in water. Under these conditions, the latter reagent, as with dihydropterins (Kawai & Scrimgeour, 1972), specifically reduces quinoid 6-Me-PDH_2 to 6-Me-PDH_4 without affecting 6-Me-7,8-PDH_2. The dithionite-treated samples were analyzed by HPLC on R-Sil SCX, 25 × 0.4 cm, eluted with 80% ammonium acetate (0.2 M in ammonia), pH 4.8, containing 1.0 mM Na_2EDTA/20% methanol at 2 mL/min. The absorbance of both 6-Me-7,8-PDH_2 and 6-Me-PDH_4 was monitored at 254 nm. In addition, the latter compound was detected by electrochemical oxidation at 0.3V vs. Ag/AgCl.

The overall first-order rate of loss of quinoid 6-Me-PDH_2, as reflected by the recovered 6-Me-PDH_4, was 0.0017 s^{-1} ($t_{1/2}$ = 6.8 min). The yield of 6-Me-7,8-PDH_2 was 53 ± 5%, giving a tautomerization rate of 9×10^{-4} s^{-1}. The competing pathway leading to decomposition of quinoid pyrimidodiazepine appears to proceed, at least in part, by ring opening at the C4a–N5 bond. This is indicated by the nature of the major byproduct, which matches the retention characteristics [Rosil ODS 5 μm, eluted with 90% sodium phosphate (0.1 M in sodium), pH 2.6, including 3 mM sodium octanesulfonate/10% methanol], and absorption spectrum (λ_{max} at 204 nm, shoulder at 235 nm in the above pH 2.6 elution buffer) of the major decay product of the ring-opened quinoid (IIIb). Further, the rate of ring opening is increased in acid to the exclusion of tautomerization. At pH 1, the quinoid IIIb itself is observed as the major product of quinoid 6-Me-PH_2 decay. Accumulation of IIIb is also facilitated by its increased stability in acid.

Tautomerization of Quinoid 6-Methyldihydropterin. The yield of 6-Me-7,8-PH_2 obtained from the rearrangement of quinoid 6-Me-PH_2 was determined by a procedure that is not dependent on the extinction coefficients of starting material or product. Quinoid 6-Me-PH_2 was prepared by addition of 0.1 mL of 1.0 M Tris-HCl, pH 7.4, at 27 °C and 0.1 mL of 0.5 mM I_2 in water to 0.8 mL of 0.0625 mM 6-Me-PH_4 in water equilibrated to 27 °C. At 20-min intervals (the half-life of tautomerization in this environment), 100-μL aliquots were removed from the reaction and added to a 100-μL solution containing 1.0 unit of dihydropteridine reductase, 0.4 mM NADH, 0.35 unit of dihydrofolate reductase, and 0.4 mM NADPH in 0.1 M Tris-HCl, pH 7.4, at 27 °C. The mixture was incubated a further 5 min at 27 °C by which time any quinoid 6-Me-PH_2 and/or 6-Me-7,8-PH_2 were reduced. The samples were put on ice and analyzed without deproteinization on Partisil SCX (25 × 0.4 cm) eluted with ammonium formate (0.1 M in ammonia), pH 3.3, containing 1 mM Na_2EDTA, with detection by electrochemical oxidation at 0.3 V vs. Ag/AgCl and absorbance at 340 nm. As tautomerization proceeded to completion, the recovered 6-Me-PH_4 decreased, reaching a plateau of 91% after 2 h. The total recovery, including a 4% yield of parent 6-Me-P, was 95 ± 2%.

The non-pterin products accompanying tautomerization of quinoid 6-Me-PH_2 can be monitored by HPLC on Rosil ODS, 5 μm, eluted with 90% sodium phosphate (0.1 M in sodium), pH 2.6, including 3 mM sodium octanesulfonate/10% methanol with detection by absorbance at 230 nm. The degradation pathway(s) was (were) emphasized through use of quinoid 6-deuterio-6-methyl-PH_2. The major byproducts that develop upon incubation of quinoid 6-D-6-Me-PH_2 in 0.1 M Tris-HCl, pH 7.4, at 27 °C for 7 h (twice the tautomerization half-life) do not match, by comparison of retention times, the major byproducts left upon completion of cyclization (at 2 min) of IIIa in the same buffer. The contribution of ring opening at C4a–N5 to the decomposition of this quinoid pterin at neutral pH is thus unclear. However, as the pH is lowered, the chromatographic profiles of their respective decay products begin to shift and converge. By pH 3.5, all of the major peaks resulting from a 5-h incubation of the quinoid dihydropterin correspond in retention and in UV absorption spectra to those in a 20-min incubation of IIIa. This match is not due to

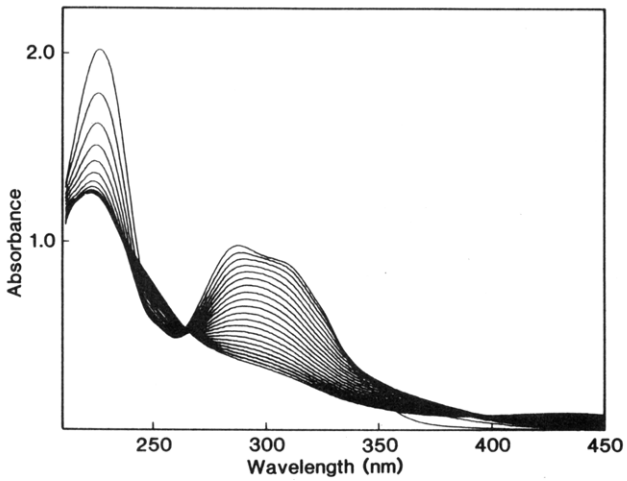

FIGURE 6: Autoxidative decomposition of 6-Me-7,8-PDH_2 in 0.1 M Tris-HCl, pH 7.4, at 27 °C; 10 min between scans.

formation of quinoid 6-Me-PH_2 from IIIa, since there is an approximate 100-fold decrease at this pH in the rate of cyclization. A spectrum similar to that of the dihydroimidazopyrazine observed in the decay of quinoid 6,6,7,7-tetramethyldihydropterin (Eberlein et al., 1984) was not seen among the major peaks of decomposing quinoid 6-D-6-Me-PH_2 in either the neutral or acidic buffers.

Ring Opening Decomposition of 6-Me-7,8-PDH_2. The behavior of the 7,8-dihydro species of pyrimidodiazepine in neutral aqueous solution differs markedly from that of the homologous pterin, which oxidizes to 6-Me-P with relatively clean isosbestic points in approximately 1 day in dilute solution. In contrast, a 0.1 mM solution of 6-Me-7,8-PDH_2 in 0.1 M Tris-HCl, pH 7.4, at 27 °C undergoes a complex series of spectral shifts, resulting in the eventual loss of most mid- and long-wave ultraviolet absorbance (Figure 6). The time to half-loss of 6-Me-7,8-PDH_2 determined by cation-exchange chromatography [Rosil SCX, 25 × 0.4 cm, eluted with 80% ammonium acetate (0.2 M in ammonia), pH 4.8, containing 1.0 mM EDTA/20% methanol at 2 mL/min] is about 60 min. No decay is observed in totally anaerobic buffer or in the presence of 50 mM 2-mercaptoethanol. After 1 or 2 h, the spectrum of autooxidizing 6-Me-7,8-PDH_2 is very similar to those of XVa and XVb. The transformation of 6-Me-7,8-PDH_2 to a compound with this same quinoid-like absorbance with a λ_{max} of 295 nm (at pH 7.4) is rapidly catalyzed by peroxidase and H_2O_2 (Figure 7). Whether produced enzymatically or by autoxidation, this intermediate further decays with a first-order half-life of approximately 100 min to a spectrum lacking significant absorbance above 260 nm. If the immediate product of peroxidase and H_2O_2 is acidified to pH 3, a spectrum similar to either IIIa or IIIb at this pH rapidly appears with λ_{max} at 223 and 271 nm. HPLC of partially air-oxidized 6-Me-7,8-PDH_2 with an acidic eluant shows a major peak with these same absorbance characteristics. Upon reneutralization, the similarity to IIIa and IIIb is maintained with λ_{max} at 244 and 282 nm. However, as with IIIb, this compound is very unstable, collapsing altogether in the mid ultraviolet over the course of a few minutes.

Substrate Properties of Quinoid 6-Me-PDH_2 Compared to Quinoid 6-Me-PH_2 for Dihydropteridine Reductase (DHPR). The enzyme-dependent rate of reduction of the two substrates was measured by monitoring NADH consumption at 340 nm under conditions maintaining quinoid concentration. Reactions containing Tris-HCl buffer, 8 units of peroxidase, and 15 milliunits of DHPR were incubated for 4 min at 27 °C, at which time H_2O_2 was added (final concentration 0.4 mM).

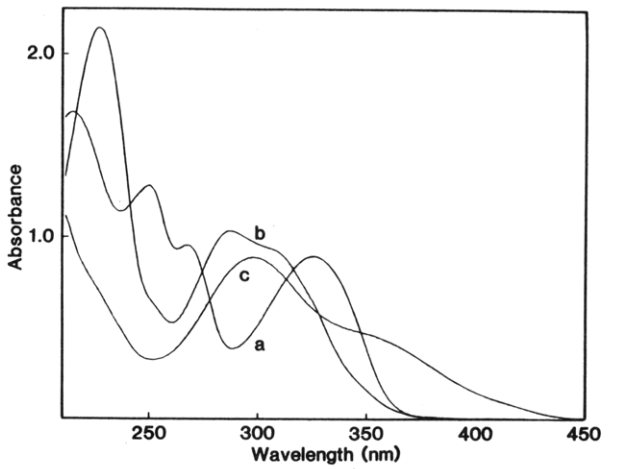

FIGURE 7: Spectra of 1.0×10^{-4} M 6-Me-7,8-PDH_2 in (a) 0.1 N HCl, (b) 0.1 M Tris-HCl, pH 7.4 (same as the first scan in Figure 6), and (c) 0.1 M Tris-HCl, pH 7.4, including 6 units of peroxidase and 3×10^{-4} M H_2O_2, scanned 3 min after addition of 6-Me-7,8-PDH_2.

At 5 min, the reaction was initiated with 0.2 μmol of NADH and either 6-Me-PDH_4 or 6-Me-PH_4, for a final volume of 1.0 mL of 0.1 M pH 7.4 Tris-HCl buffer. The reference was identical except that DHPR was omitted. Linear rates were observed for several minutes. The K_m of the quinoid dihydropyrimidodiazepine was 33 ± 8 μM and was not significantly different from the K_m for quinoid 6-MePH_2 (Bailey & Ayling, 1983, Armarego et al., 1984). The V_{max} with quinoid 6-Me-PDH_2 was also very close to that with quinoid 6-Me-PH_2.

Comparison of 6-Me-7,8-PDH_2 and 6-Me-7,8-PH_2 as Substrates for Dihydrofolate Reductase. The background rate of a mixture consisting of the 7,8-dihydro compound with 100 μM NADPH in 0.1 M Tris-HCl, pH 7.4, at 27 °C was monitored spectrally at 340 nm. The complete reaction was then initiated by addition of either 250 milliunits or, in the case of 7,8-dihydrofolate, 5 milliunits of dihydrofolate reductase to make a final volume of 1.0 mL. The contribution to the total absorbance change of the reaction at 340 nm due to reduction of either 6-Me-PDH_2 or 6-Me-PH_2 was determined to be 2200 M^{-1} cm^{-1} and 3800 M^{-1} cm^{-1}, respectively, in addition to 6220 M^{-1} cm^{-1} for the oxidation of NADPH to NADP. A rate in the absence of enzyme was observed only with the pyrimidodiazepine, due to the maintenance of this compound against autooxidation (see decomposition of 6-Me-7,8-PDH_2 above) at the expense of NADPH. The K_m's for 6-Me-7,8-PH_2 and 6-Me-7,8-PDH_2 were found to be 0.10 mM and 0.16 mM, with relative maximum velocities of 0.14 and 0.1, respectively, of that seen with a saturating (60 μM) concentration of 7,8 dihydrofolate.[3]

Kinetic Parameters of 6-Me-PDH_4 with Phenylalanine Hydroxylase. The rates of enzymatic tyrosine formation vs. varying concentration of either pyrimidodiazepine cofactor, phenylalanine, or oxygen were measured in reaction mixtures having the following common components: 0.1 M Tris-HCl, pH 7.4, at 27 °C, 1.0 mM Na_2EDTA, 60 units of superoxide dismutase, 600 units of catalase, and purified phenylalanine hydroxylase. When oxygen K_m's were measured, nonenzyme

[3] The value reported for the K_m of 6-Me-7,8-PH_2 at pH 6.5 with rat liver dihydrofolate reductase is 0.01 mM, with a maximum velocity relative to dihydrofolate of 0.25 (Webber & Whiteley, 1985). These differences from the values reported here are primarily due to the pH, since at pH 6.5 the K_m for 6-Me-7,8-PH_2 with the beef liver enzyme was found to be 0.018 mM, with a maximum velocity relative to dihydrofolate of 0.3 (J. E. Ayling and S. B. Dillard, unpublished results).

Table I: Effect of Expanding Cofactor Ring Size on Kinetic Parameters of Phenylalanine Hydroxylase[a]

	6-Me-PH_4	6-Me-PDH_4
K_m' for cofactor (mM)	0.1	0.15
K_m' for phenylalanine (mM)	0.3	0.44
K_m' for oxygen (%)	<5	58
V_{max}	1950	77–134

[a] V_{max} is expressed as nmol of tyrosine produced min^{-1} (unit of phenylalanine hydroxylase)$^{-1}$, calculated for saturation with phenylalanine, oxygen, and cofactor, where the range indicates the range of uncertainty in the effect of oxygen concentration on cofactor K_m.

Table II: Effect of 5-Amino Substituent of Pyrimidine Cofactors on Kinetic Parameters of Phenylalanine Hydroxylase[a]

	TP	5N-Bz-TP	5N-Me-TP
K_m' for cofactor (mM)	0.1	0.003	0.13
K_m' for Phe (mM)	0.1	0.33	0.46
K_m' for oxygen (%)	10.5	210	132
V_{max}	22–33	170	2.8–4.8

[a] See legend to Table I.

components were first equilibrated to the oxygen/argon mixture flowing through the reaction chamber head space before addition of enzymes in a minimum volume. All reaction mixtures were incubated for 5 min at 27 °C before initiation with cofactor. Cofactor was added in a minimum volume of deoxygenated water. The final reaction volume was 0.25 mL. Samples were withdrawn immediately and at 2 and 4 min, each being diluted with an equal volume of 1.0 M trichloroacetic acid to stop the reaction. The tyrosine produced was assayed by HPLC with fluorescence detection (Bailey & Ayling, 1980a). The resulting apparent K_m's and V_{max} for 6-Me-PDH_4 are compared with those for 6-Me-PH_4 in Table I.

The extra methylene group in the ring of 6-Me-PDH_4 has very little effect on the K_m for phenylalanine or the apparent K_m for cofactor, although the apparent K_m for O_2 is increased by more than 10-fold. The maximum velocity with 6-Me-PH_4, calculated for enzyme saturated with all three substrates, is 1950 nmol of tyrosine formed min^{-1} (unit of phenylalanine hydroxylase)$^{-1}$. By comparison, the V_{max} similarly calculated for 6-Me-PDH_4 is 77–134 nmol of tyrosine min^{-1} (unit of enzyme)$^{-1}$, a factor of 15–25 less.

Reaction of Phenylalanine Hydroxylase with Pyrimidine Cofactors. We have previously reported the apparent maximum velocities and Michaelis constants for 2,5,6-triamino-4(3*H*)-pyrimidinone (TP) (V, R = H) and 2,6-diamino-5-(benzylamino)-4(3*H*)-pyrimidinone (5N-Bz-TP) (V, R = benzyl) measured at 1 mM phenylalanine and atmospheric oxygen (Bailey & Ayling, 1978). In order to compare the k_{cat}'s of pyrimidine cofactors, the K_m's for oxygen and phenylalanine, as well as for cofactor, have been determined with TP, 5N-Bz-TP, and 2,6-diamino-5-(methylamino)-4(3*H*)-pyrimidinone (5N-Me-TP) (V, R = methyl).

Assay conditions were similar to those for determination of the kinetic parameters of 6-Me-PDH_4. The results, summarized in Table II, indicate that the maximum rate of enzymatic hydroxylation of phenylalanine calculated for saturation with all three substrates is greatly influenced by the nature of the substituent on the 5-amino group of the pyrimidine cofactor.

Discussion

Seven-membered cyclic hydrocarbons are usually more strained than their six-membered counterparts. Several results of this investigation indicate that expansion of the pyrazine ring in a pterin with a methylene group also leads to increased strain. The first is the comparison of the ring closure of quinoid 6-*N*-(2′-aminopropyl)divicine (IIIa) with that of the 3′-aminobutyl homologue (IIIb). Like unsubstituted quinoid divicine (Bailey et al., 1982), both are readily obtained by oxidation of the appropriate 6-*N*-(aminoalkyl) derivatives of 2,5,6-triamino-4(3*H*)-pyrimidinone (XIVa) and XIVb in 0.01 N HCl. Dilution of IIIa into neutral buffer initiates a rapid intramolecular condensation to quinoid 6-Me-PH_2 via the C4a–N5 hydrate. In contrast, cyclization of IIIb to quinoid 6-Me-PDH_2 could not be detected. The rate of formation of the C4a-hydroxy adduct (IVa) from IIIa is pH-dependent in a manner controlled primarily by the charge on the terminal amino group. As the reaction buffer is made more alkaline, the yield of quinoid 6-Me-PH_2 (or the subsequent 6-Me-7,8-PH_2 tautomer) climbs, reaching a maximal value between pH 8.5 and 9.5 (S. W. Bailey and J. E. Ayling, unpublished results). In 0.1 M Tris-HCl, pH 7.4, the yield of 57% is somewhat less than maximal. With Tris buffers, this physiological pH is only slightly above the point at which the rate of dehydration of the carbinolamine intermediate IVa equals its formation. Some of these results are qualitatively similar to those reported for 6-*N*-(2′-amino-1′-methylpropyl)-TP (Lazarus et al., 1981). The higher yield and difference in effect of pH in the current study are due primarily to the generation of the quinoid divicine derivative (IIIa) in a discrete acid-catalyzed step prior to cyclization.

The lack of condensation of the higher homologue is probably due to (a) extra strain and entropy loss in the transition state decreasing the rate of closure to the point where decomposition of starting material is dominant and/or (b) alteration of the equilibrium between IIIb and quinoid 6-Me-PDH_2 by the additional strain of a seven-membered ring. The recovery of a small amount (2.2%) of 6-Me-PDH_4 upon trapping the initial C4a-hydroxy adduct with PHS and DHPR/NADH shows that the rate of ring closure is indeed markedly decreased, though not entirely eliminated. Since the rates of decomposition of both IIIa and IIIb in Tris-HCl, pH 7.4, are identical, the difference in yields implies a 26-fold slower cyclization of the seven-membered ring.

$$\text{decay} \xleftarrow{0.043\ s^{-1}} \text{IIIa} \underset{}{\overset{0.056\ s^{-1}}{\rightleftharpoons}} \text{IVa} \overset{0.036\ s^{-1}}{\rightleftharpoons} \text{quinoid 6-Me-}PH_2$$

$$\text{decay} \xleftarrow{0.043\ s^{-1}} \text{IIIb} \overset{\sim 0.0022\ s^{-1}}{\rightleftharpoons} \text{IVb}$$

This ratio, which should be considered a maximum value since the efficiency of the trapping method was not determined, implies a 2 kcal M^{-1} increase in ΔG^* of the cyclization transition state.

The position of the equilibrium between quinoid 6-Me-PH_2 and IIIa is beyond current measurement limits (5.1 kcal M^{-1}). Whether the equilibrium between quinoid 6-Me-PDH_2 and IIIb is shifted enough to favor the latter is, therefore, difficult to assess. Since the difference in the entropy of cyclization of six- vs. seven-membered hydrocarbons is small (Page & Jencks, 1971; Page, 1973; Capon & McManus, 1976), strain alone may be the most significant factor contributing to the disadvantage of forming a pyrimidodiazepine over that of a pterin. The strain differences between cyclohexane vs. cycloheptane, cyclohexene vs. cycloheptene, and methylenecyclohexane vs. methylenecycloheptane range between 4 and 6 kcal·M^{-1} (Schleyer et al., 1970; Allinger et al., 1972; White & Bovill, 1977). Empirical comparison of azacycloalkanes with cycloalkanes shows very similar strain energies (Pell & Pilcher, 1965), suggesting that the above range of values can be applied as a first approximation. Most of this predicted

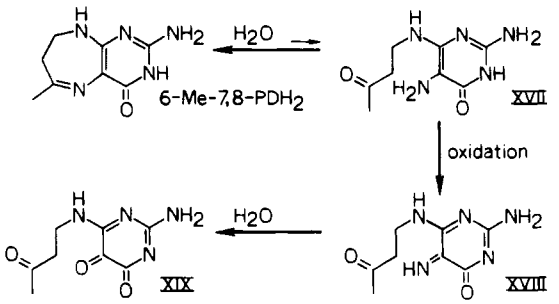

FIGURE 8: Proposed pathway for the autoxidative decomposition of 6-Me-7,8-PDH_2 via hydrolysis of the 5,6-imine bond.

increase in ΔG of cyclization of IIIb would be expected to appear in the equilibrium with the carbinolamine intermediate. The fraction of additional strain that is transmitted to the transition state in the initial stage of condensation will diminish the rate of cyclization, as observed.

The effect of the extra methylene group in a pyrimidodiazepine can also be seen in the instability of the quinoid form to ring opening (the reverse of the above reaction). The current findings demonstrate that even the conversion of quinoid 6-Me-PH_2 to 6-Me-7,8-PH_2 is not completely quantitative (95%). The lack of full recovery is accentuated in the tautomerization of the more strained quinoid 6-Me-PDH_2, which gives only 53% yield of 6-Me-7,8-PDH_2:

$$\text{unknown products} \xleftarrow{0.0017\ \text{min}^{-1}} \text{6-Me-}PH_2 \xrightarrow{0.033\ \text{min}^{-1}} \text{6-Me-7,8-}PH_2$$

$$\text{decay via IIIb} \xleftarrow{0.049\ \text{min}^{-1}} \text{6-Me-}PDH_2 \xrightarrow{0.054\ \text{min}^{-1}} \text{6-Me-7,8-}PDH_2$$

The sum of the decomposition pathways of the quinoid pyrimidodiazepine is, therefore, 28 times faster than in the quinoid pterin.

The byproducts of the tautomerization of quinoid 6-Me-PDH_2 at neutral pH appear to be generated by an initial hydrolytic cleavage of the C4a–N5 bond, since chromatographically and spectrally identical compounds result from the decomposition of IIIb. Direct evidence for a ring opening mechanism of quinoid 6-Me-PDH_2 decay is the recovery of IIIb itself in acidic conditions. This is probably due, in part, to a shift in the rate-limiting step of imine hydrolysis with acid catalysis (Jencks, 1969). In contrast, although the pattern of decay products of quinoid 6-D-6-Me-PH_2 closely resembles that of IIIa at pH 3.5, no correspondence at pH 7.4 was seen. The more conspicuous decomposition via ring opening at neutral pH of the quinoid pyrimidodiazepine compared to the quinoid pterin could be due to a greater propensity for hydration and/or increased rate of subsequent cleavage.

The final indication of increased strain in the pyrimidodiazepine family is the comparative instability of 6-Me-7,8-PDH_2. Whereas, 6-methyl-7,8-dihydropterin slowly oxidizes to the aromatic 6-methylpterin, the ring-expanded homologue decomposes much more rapidly to a mixture of compounds that absorb primarily in the far ultraviolet (Figure 6). A pathway for the decomposition of 6-Me-7,8-PDH_2 that is consistent with the UV spectra of the intermediates is proposed in Figure 8. The pyrimidodiazepine, like 7,8-dihydropterin (Armarego et al., 1983) is probably in equilibrium with trace amounts of the N5–C6 hydrate. Unlike 6-Me-7,8-PH_2, however, the more strained seven-membered ring partially opens to 2,5-diamino-6-[(3′-ketobutyl)amino]-4(3*H*)-pyrimidinone (XVII). The small equilibrium quantity of XVII is then subject to autooxidation, or the action of H_2O_2/peroxidase, resulting eventually in the complete conversion to the quinoid XVIII. This intermediate, the spectrum of which (Figure 7c) matches that of XVa and XVb, eventually decomposes further via the quinoid divicine derivative (XIX), to non-pyrimidine products.

The pyrazine ring cleavage by phenylalanine hydroxylase proposed by Hamilton was probed as a possible rate-limiting step with 6-Me-PDH_4. It has been suggested that entropy of activation does not play a major role, in comparison to strain, in determining the relative rates of ring opening in a series of varying ring size (Cerichelli et al., 1980). Although counterexamples are known (e.g., the hydrolysis of cyclic sulfonate esters; Laleh et al., 1980), the generalization is probably applicable to the opening of a cofactor hydroperoxide, since little rearrangement in the transition state is expected. Considering the apparent strain displayed by quinoid 6-Me-PDH_2 and 6-Me-7,8-PDH_2, it is likely that strain in the putative C4a-hydroperoxide of the pyrimidodiazepine (Ib, Figure 1) would be greater than that of the equivalent pterin (Ia). If the carbonyl oxide (IIa,b) is the ultimate form of activated oxygen in phenylalanine hydroxylation and if generation of this reagent by cleavage of the C4a–N5 bond is rate-limiting, a faster reaction would be expected from 6-Me-PDH_4 than from 6-Me-PH_4. The magnitude of the increase would depend on the extent to which the extra strain in the pyrimidodiazepine hydroperoxide is transmitted to the transition state. In ring opening substitution of *N*,*N*-dimethylcycloazaalkane iodides with methoxide ion, the seven-membered ring reacted 2.4 times faster (Cerichelli et al., 1980). Instead, the enzymatic utilization of 6-Me-PDH_4 was found to be 15–25 times slower than that of 6-Me-PH_4 when compared under saturating concentrations of cofactor, phenylalanine, and O_2. Both reactions yield one tyrosine per cofactor oxidized (S. W. Bailey and J. E. Ayling, unpublished results). The maximum rate with the pyrimidodiazepine is faster than that of the prototypic pyrimidine cofactor 2,5,6-triamino-4(3*H*)-pyrimidinone by only a factor of around 4. Therefore, unless ring opening is facilitated by an enzyme-induced strain not available to the diazepine, this cleavage, if it occurs at all, is not the rate-limiting step.

The utilization of 2,5,6-triamino-4(3*H*)-pyrimidinone, or its 5-*N*-benzyl or 5-*N*-methyl derivative, by phenylalanine hydroxylase results not only in tyrosine formation but also in the cleavage of the cofactor into quinoid divicine plus ammonia, benzylamine, or methylamine, respectively (Bailey & Ayling, 1980b). Whether this is the result of Hamilton's proposed mechanism or of preferential loss of the amine from a carbinolamine subsequent to substrate hydroxylation is currently unknown. With this class of cofactors, loss of the 5-amino group from the hydroperoxide intermediate (VI, Figure 2) would leave an unvarying structure, the carbonyl oxide of quinoid divicine (VII) (Bailey & Ayling, 1980b), regardless of any substitution of that amine. If utilization of this oxene reagent is rate-limiting, the maximum rate of hydroxylation should be independent of the substituent on the pyrimidine 5-amino group. Comparison of these three pyrimidine cofactors at saturating concentrations of cofactor, phenylalanine, and oxygen gave a V_{max} with the 5-*N*-benzyl derivative that is at least 5-fold faster and with the 5-*N*-methyl derivative at least 5 times slower than that of TP. The 35–60-fold range of rates between the fastest and the slowest demonstrates that the hypothesized transfer of oxygen from a carbonyl oxide to phenylalanine is also not rate limiting. Further, the critical step cannot occur later in Hamilton's sequence. Thus, the continued viability of the carbonyl oxide

as an intermediate in pteridine-dependent monooxygenases requires that the rate limitation occur earlier than cleavage of the C4a–N5 bond.

The properties of the strained pyrimidodiazepine cofactor provide further opportunities for the investigation of the nature of the cofactor/oxygen adduct formed by phenylalanine hydroxylase. The above results show that synthetic quinoid 6-*N*-(3′-aminobutyl)divicine (IIIb), the hypothetical product of Hamilton's pathway as applied to 6-Me-PDH_4, does not spontaneously close to quinoid 6-Me-PDH_2 in enzyme assay conditions. If the ring-opened compound is the product of the hydroxylase reaction, it should not be possible to use 6-Me-PDH_4 catalytically in the presence of a cofactor regeneration system. The results of an investigation of this issue will be the subject of a future paper.

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Registry No. IIIa, 103422-07-7; IIIb, 103422-08-8; IVa, 83387-39-7; IVb, 103422-09-9; IX, 3783-77-5; X, 74274-12-7; XI, 103437-37-2; XII, 103422-10-2; XIIIa, 103422-11-3; XIIIb, 103422-12-4; XIVa, 103422-13-5; XIVb, 103422-14-6; XVIa, 103437-38-3; 6-Me-7,8-PDH_2, 96487-57-9; XVIb, 103422-15-7; 6-Me-PDH_4, 103422-16-8; 6-Me-PH_4, 942-41-6; 6-Me-7,8-PH_2, 17377-13-8; TP, 1004-75-7; DHFR, 9002-03-3; DHPR, 9074-11-7; 5-*N*-Bz-TP, 25468-62-6; 5-*N*-Me-TP, 1688-92-2; quinoid-6-D-6-Me-PH_2, 103422-18-0; quinoid-6-Me-PDH_2, 103422-17-9; quinoid-6-Me-PH_2, 70786-93-5; O_2, 7782-44-7; L-Phe, 63-91-2; Cl-$(CH_2)_2$COMe, 6322-49-2; $NH_2(CH_2)_2CH(NH_2)$Me, 590-88-5; potassium phthalimide, 1074-82-4; 2-amino-6-chloro-5-nitro-4(3*H*)-pyrimidinone, 1007-99-4; phenylalanine hydroxylase, 9029-73-6.

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