Synthesis of 4a-Hydroxytetrahydropterins and the Mechanism of Their Nonenzymatic Dehydration to Quinoid Dihydropterins

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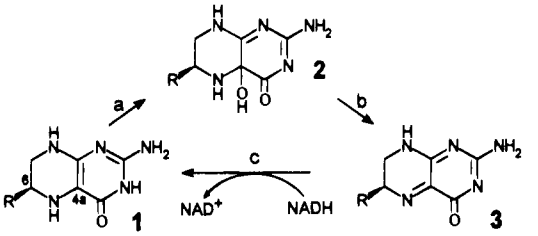
Abstract: 4a-Hydroxytetrahydropterins **2** (R = Me, Pr, and 1′(*R*),2′(*S*)-dihydroxypropyl) were synthesized by intramolecular Schiff base condensation of 2′-substituted 2-amino-6-[(aminoethyl)amino]-4,5-pyrimidinediones **6**. The rate vs pH of cyclization of **6** (which is predominately monohydrated) follows a bell-shaped curve with maxima at pH 9.8 in H_2O (consistent with p*K* values of 8.8 and 10.8) and pH 9.1 in MeOH. Although almost insignificant in organic solvents, substantial 7-substituted dihydropterin was generated in water, particularly with **6** (R = dihydroxypropyl). The minimum rate of dehydration of carbinolamine **2** (R = Me or Pr) in "zero" buffer (0.0035 s^{-1} at 17 °C) occurs at pH 8.25, and is catalyzed by proton (1.3×10^5 M^{-1} s^{-1}) and general acids. Between pH 8.4 and pH 7.4 ΔH^* decreases from 15 to 12 kcal/mol, while ΔS^* decreases from −18 to −26 eu for **2** (R = Me or Pr), consistent with concerted proton transfer in the dehydration transition state. Surprisingly, the rate also increases in more alkaline conditions up to 12-fold (in zero buffer), coincident with formation of a pteridine anion (p*K* = 9.8). Below pH 11.5 catalysis by buffer base was also observed. A solvent kinetic isotope effect (k_{H_2O}/k_{D_2O}) of 2.6, 2.2, and 3.5 was found in dilute buffers at pH 7.4, 8.2, and 10.9, respectively. The overall rate of disappearance of **2** (R = dihydroxypropyl) is similar to that of the alkyl analogs, but a second pathway competes with dehydration to produce a compound tentatively identified as a side-chain 4a-cyclic adduct, which subsequently decays to quinoid 6(*R*)-dihydrobiopterin. These synthetic substrates have permitted the first kinetic characterization of 4a-hydroxytetrahydropterin dehydratase (Rebrin, I.; et al. *Biochemistry* **1995**, *34*, 5801−5810), the enzyme involved in cofactor regeneration during aromatic amino acid hydroxylation.

Introduction

For many years it was generally believed that the product of utilization of 6(*R*)-tetrahydrobiopterin **1** (R = 1′(*R*),2′(S)-dihydroxypropyl) by the aromatic amino acid hydroxylases is a 2-electron-oxidized form identified as having quinoid structure **3**[1] (Scheme 1). Since the latter quinoid is efficiently reduced by dihydropteridine reductase, the understanding of cofactor regeneration at the expense of NADH and/or NADPH seemed complete. This view was then challenged by the observation of an unstable intermediate, which was proposed to be 4a-hydroxytetrahydrobiopterin **2** (R = 1′,2′-dihydroxypropyl) (i.e., the 4a,*N*5-hydrate of quinoid dihydrobiopterin) on the basis of its UV spectrum.[2,3] That this is indeed the case was then demonstrated by ^{18}O labeling of pyrimidine cofactor analogs[4] and ^{13}C-NMR studies of tetrahydropterin[5] cofactor analogs.[6,7] Further, the stimulation of phenylalanine hydroxylase activity by a protein isolated from liver[8] was later shown to be due to its ability to catalyze the dehydration of 4a-hydroxytetrahydropterins.[9] Interestingly, the protein possessing this dehydratase activity was found to have a sequence identical to that of DCoH, a regulator of the hepatic nuclear-transcription factor 1α.[10,11] Examination of the dehydratase mechanism has been hindered by the lack of understanding of the chemistry of 4a-hydroxytetrahydropterins. This in turn is primarily due to the lack of a synthetic procedure for generating these unstable compounds. While some properties of 4a-hydroxytetrahydropterins generated by phenylalanine hydroxylase have been examined,[6,12,13] this method is too limited to permit thorough study of 4a-carbinolamine chemistry, let alone dehydratase kinetics.

Scheme 1. Regeneration of Tetrahydropterin Cofactor **1**, after Oxidation to **2** by Aromatic Amino Acid Hydroxylases (a) Occurs via Action of 4a-Hydroxytetrahydropterin Dehydratase (b) and Subsequent Reduction by Dihydropteridine Reductase (c) (Tautomeric Structure of **2** Not Yet Known)



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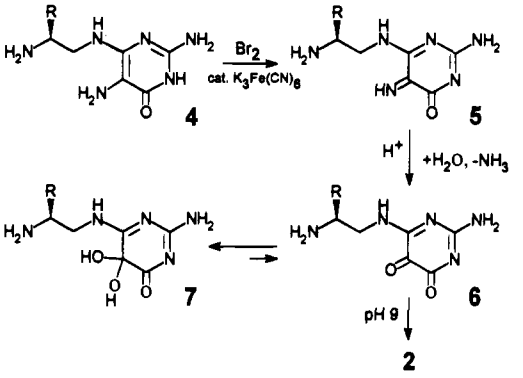
Previously, we have shown that tetrahydropterins can be stereospecifically synthesized by an intramolecular condensation of 2′-derivatives of quinoid N^6-(2′-aminoethyl)divicine **6** via a presumed 4a-hydroxytetrahydropterin intermediate.[14] A procedure has been developed from this earlier work that for the first time generates 4a-hydroxytetrahydropterins in sufficient quantity and purity to allow measurements of the effects of pH, buffer catalysis, temperature, solvent kinetic isotope effect, and pteridine structure on the rate of nonenzymatic dehydration. In comparison to previous studies of carbinolamine dehydration,[15] several surprising features of this reaction were observed including an unexpected susceptibility to catalysis by base. The synthesis and chemical characterization of the unstable 4a-hydroxytetrahydropterins are the subject of this paper.[16,17]

Scheme 2



Experimental Section

General Procedures. All buffers were made to be the desired pH at each temperature as measured with an Orion 81-75 electrode. The pH of D_2O (99.7% D, final) solutions was determined by adding 0.4 to the meter reading.[18] Borate buffer concentrations are expressed in terms of total boron, i.e., as if made from boric acid. All solvents were HPLC grade and were used without further purification or drying. Reactions monitored spectrophotometrically (e.g., cyclization and dehydration) were performed in a Precision Cells Type 51 water-jacketed quartz cuvette maintained to within 0.15 °C as measured by a YSI Type 729 thermistor probe. Typical reactions were initiated by transfer of 10 μL of sample from a dry-ice bath using a Rainin positive displacement pipet into 0.99 mL of temperature-equilibrated buffer and mixed with a low thermal mass mixing rod. The resulting absorbance changes were digitally recorded with a Nelson 3000 data system. All data fitting was performed using MINSQ version 4.0 or Scientist II from MicroMath (Salt Lake City). Mass spectra were acquired on a VG70-250SEQ with a continuous FAB probe and a xenon beam. Samples were injected into a stream of 10% glycerol (5 μL/min) via a Rheodyne valve.

HPLC. 4a-Hydroxy-6(*S*)-methyltetrahydropterin, quinoid 6- and 7-methyldihydropterins, and 4a-hydroxy-6(*R*)-tetrahydrobiopterin were chromatographed on Hypersil BDS-C_{18},[19] 3 μm (5.0 × 0.46 cm column plus 1 cm guard), eluted at 1.0−1.5 mL/min with 10 mM Tris[20] plus acetic acid to pH 8.2 at 0 °C. The 6-propyl-substituted compounds were chromatographed similarly except 10% methanol was included in the eluant. When increased resolution was required, 4a-hydroxy-6(*R*)-tetrahydrobiopterin was chromatographed on a 25 × 0.46 cm column with a 2 × 0.21 cm guard of 5 μm Kromasil C_{18}, which is more stable to dissolution at pH 8.2 than Hypersil. A 20 cm coil of stainless steel capillary preceded the guard and main columns which all together were placed in a jacket circulated with water at 0 °C. Detection was with a Waters Associates Model 996 photodiode array spectrometer, and amperometrically using a potentiostat incorporating BAS MF1000 glassy carbon and MF1018 Ag/AgCl reference electrodes. Samples of 4a-hydroxytetrahydropterins were diluted 20-fold into 10 mM Tris−HCl, pH 8.2, at 0 °C immediately prior to analysis to avoid loss of resolution caused by direct injection of methanol solutions. Carbinolamine dehydration in the above chromatographic systems is quite low, though detectable with the diode array spectrometer. Cyclization yields of 6(*S*)-methyltetrahydropterin were determined by strong cation exchange chromatography as described earlier for 6(*S*)-propyltetrahydropterin[14] except that methanol was decreased to 7%.

Materials. 7(*R,S*)-Tetrahydrobiopterin was obtained from B. Schircks (Jona, Switzerland); 7(*R,S*)-methyltetrahydropterin was made by catalytic reduction[21] of 7-methylpterin.[22] (*R,S*-Methyl-6-phenyltetrahydropterin and quinoid (*R,S*)-6-methyl-6-phenyldihydropterin were synthesized by modification of a published procedure for 6,6-dimethyltetrahydropterin[23] to be reported elsewhere. Phenylalanine hydroxylase, dihydropteridine reductase, and 4a-hydroxytetrahydropterin dehydratase were purified and assayed as previously reported.[24]

Oxidation and Hydrolysis of 2,5-Diamino-6-[[2′(*S*)-amino(propyl or pentyl)]amino]-4(3*H*)-pyrimidinones 4 (R = Me or Pr) (Scheme 2). To 2.0 mL of a 10 mM solution[25] of **4**·3HCl[14] in methanol[26] at 27 °C were added with good stirring 40 μL of 0.5 M Br_2 (freshly made in ice-cold methanol) and 10 μL of 0.05 M $K_3Fe(CN)_6$ (freshly made in H_2O) (the two oxidants being premixed immediately prior to use). The nearly colorless solution was kept at 27 °C for 2.0 min and then transferred to a dry-ice/EtOH bath.[27] This solution of quinoid divicine derivative **6** and its hydrate **7** is stable for several hours at −78 °C. Accurate FAB mass spectrometry of an 80 mM solution of **6** and **7** (R = Me) gave peaks for both species at *m/z* 216.113 (calcd for $C_7H_{14}N_5O_3$, 216.110) and *m/z* 198.103 (calcd for $C_7H_{12}N_5O_2$, 198.099) in a ratio of **7** to **6** of about 4:1. No significant signal for the carbinolamine intermediate between **4** and **6**, *m/z* 215, was detected. The quinoid divicine precursor to the 4a-hydroxy adduct of tetrahydrobiopterin **2** (R = 1′(*R*),2′(*S*)-dihydroxypropyl) was prepared by a different route to be published elsewhere. The solution of **6** and **7** in MeOH was titrated in a dry-ice/EtOH bath[27] to pH 9.1[28] using 1.0 M NaOH in MeOH. Back-titration, if necessary, was accomplished with concentrated HCl/MeOH (1:12). The alkaline solution of **6** and **7** can be stored frozen in liquid nitrogen for several months without cyclization or decomposition.

Cyclization of 2′-Derivatives of Quinoid N^6-(2′(*S*)-Aminoethyl)-divicine[29] 6 and 7. Carbinolamine **2** was generated by transferring an aliquot of the alkaline solution of **6** and **7** to a warmer bath for a specific time and then returning the sample to the dry-ice bath. The standard condition for cyclization of **6** and **7** (R = Me or Pr) was 6.0 min at 0 °C, and 3.0 min at 0 °C to produce 4a-hydroxy-6(*R*)-tetrahydrobiopterin. Solutions of **2** in MeOH can be kept for more than 8 h at −78 °C

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(25) Based on ϵ_{270} = 15 600 in 0.1 M HCl for 4 (R = Pr;[14] see supporting information for R= Me).
(26) The sulfate salt of 4 (R = Pr) was dissolved in MeOH/H_2O (6:1) with 3−4 equiv of HCl and warming.
(27) Absorption of CO_2 vapor by the cold MeOH solution must be avoided.
(28) pH was determined using the reading 1 min after a 10-fold dilution of a sample into water and immersion of the electrode.
(29) Divicine = 2,6-diamino-5-hydroxy-4(3*H*)-pyrimidinone; quinoid divicine = 2-e⁻ oxidized divicine, 2,6-diamino-4,5-pyrimidinedione (the tautomeric structure is not known).

without significant dehydration. ^{1}H NMR (relative to TMS) (−15 °C, MeOD-d_4, 300 MHz) for **2** (R = Me): δ 1.19 (3H, d, J = 5.5 Hz, $-CH_3$), 3.02 (1H, dd, J = 11, 13 Hz, H_{7a}), 3.45 (1H, dd, J = 5, 13 Hz, H_{7b}), 3.81 (1H, m, H_6). Diode array UV spectra were obtained from the portion of the chromatograms giving constant absorbance ratios at various wavelengths in 10 mM Tris−acetate, pH 8.2, at 1 °C: λ_{max} (ϵ, M^{-1} cm^{-1}) [$\epsilon_{\lambda max1}/\epsilon_{\lambda max2}$] (**2** (R = Me)) 247 (20 200 ± 3%), 290 (8400 ± 3%) [2.4]; (**2** (R = Pr)) 247, 290 [2.4]; (4a-hydroxytetrahydrobiopterin) 246, 288 [2.45]. The UV spectra, HPLC retention times, and patterns of dehydration of **2** (R = Me, Pr, and dihydroxypropyl) are identical to those of the immediate products of phenylalanine hydroxylase utilization of the respective tetrahydropterin cofactors **1**.

The concentration (and thus yield of cyclization) of carbinolamine in such solutions was determined by dilution of an aliquot into a 1.0 mL reaction in 0.025 M Tris−HCl, pH 8.4, at 10 °C containing 2 units of dihydropteridine reductase and 10 μmol of NADH, followed by addition of sufficient 4a-hydroxytetrahydropterin dehydratase to catalyze reaction to completion within 30 s. The initial instant decrease in absorbance at 340 nm before addition of dehydratase primarily shows the contamination with the corresponding quinoid dihydropterin. The decrease obtained after addition of dehydratase indicates the amount of 4a-hydroxytetrahydropterin present.[24] The net extinction at 340 nm for the conversion 4a-hydroxytetrahydropterin + NADH → tetrahydropterin + NAD was found to be 5700 M^{-1} cm^{-1} with **2** (R = Me). On completion of dehydration, the mixture was also analyzed for the resulting 6-methyltetrahydropterin by HPLC to confirm the total yield of cyclization.

The rate of cyclization of **6** and **7** (R=Me) as a function of pH was determined by following the decrease in absorbance at 280 nm upon 100-fold dilution of a 10 mM −78 °C MeOH stock solution into 10 mM sodium borate buffers at 1 °C. Since cyclization and subsequent dehydration are not completely separated temporally, the collected data were fitted to an equation describing the two consecutive reactions **6, 7** → **2** → **3**. The rate of dehydration (k_{dehyd}) of **2**, precyclized in MeOH as above, in 10 mM borate buffers at 1 °C at each pH was determined in a separate experiment. In addition to the cyclization (k_{cyc}) rate constant, the equation contained three additional parameters related to the extinction coefficients of the three species: absorbance = $Ae^{-k_{cyc}t} + Bk_{cyc}(e^{-k_{cyc}t} - e^{-k_{dehyd}t})/(k_{dehyd} - k_{cyc}) + C$, where $A = (\epsilon_{6,7} - \epsilon_3)D_o$, $B = (\epsilon_2 - \epsilon_3)D_o$, $C = \epsilon_3 D_o$ + offset, and $D_o = [\mathbf{6,7}]_{t=0}$.

The cyclization of quinoid divicines **6** and **7** (R = Me) was also studied in methanol as a function of pH. After titration of a solution at −78 °C with 1 M NaOH in MeOH to a pH between 8.1 and 10.5, the mixture was transferred to a −20 °C bath to initiate cyclization. Samples were removed at various times and quickly diluted into 0.025 M Tris−HCl, pH 8.4, at 1 °C. Cyclization of any remaining **6** and **7** and subsequent dehydration was observed spectrophotometrically by rapid repeat scanning from 350 to 220 nm. The ratio of absorbance in the first scan at 280 nm to absorbance at 297 nm (the isosbestic point of **2** and **3**, R = Me) was used to estimate the residual uncyclized material. This ratio as a function of time at −20 °C was fitted to a first-order decay to estimate the cyclization rate constant.

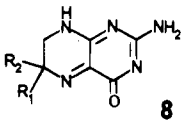
The partitioning of cyclization to 6- vs 7-substituted products was determined by 10−20-fold dilution of a MeOH solution of quinoid divicine derivatives **6** and **7** into various solvents or buffers. Upon completion of cyclization, sufficient dehydratase activity was added to aqueous reactions to promote completion of quinoid dihydropterin formation within 2 min (organic solvent reactions were diluted 20-fold further into 0.025 M Tris−HCl, pH 7.4, at 10 °C containing sufficient dehydratase). The dehydratase was used to circumvent the differential rates of formation and decay of 6- vs 7-substituted quinoid dihydropterins (**3** vs **12**). After completion of enzymatic dehydration, samples were analyzed by HPLC. Experiments with **6** and **7** (R = dihydroxypropyl) also included 2.5 mM NADH and dihydropteridine reductase with the dehydratase, and the resulting 6- and 7-substituted tetrahydrobiopterins were quantitated.

Dehydration of 4a-Hydroxytetrahydropterins 2. The rate of dehydration was determined spectrophotometrically by transferring aliquots of 4a-hydroxy-6-alkyltetrahydropterin in MeOH from a dry-ice bath into a cuvette containing temperature-equilibrated buffer. The absorbance increase was monitored at the longest wavelength isosbestic point for the subsequent tautomerization of the initially formed quinoid dihydropterin **3** to the corresponding 6-alkyl-7,8-dihydropterin. This isosbestic wavelength, which ranged from 340 nm at neutral pH to 350 nm at the most alkaline pH studied, was determined in separate experiments by repeat absorbance scans of bromine-oxidized 6-methyltetrahydropterin. In addition, the temperature dependence of dehydration at pH 7.4 and 8.4 was monitored spectrophotometrically at 340 nm by coupling the reaction to oxidation of NADH (initially 10 mM) using dihydropteridine reductase (2 units/mL of reaction). Consumption of NADH due to the autooxidation of tetrahydropterin which accumulated toward the end of the reaction did not significantly interfere. Examination of the dehydration of 4a-hydroxy-6(*R*)-tetrahydrobiopterin spectrophotometrically only employed the NADH-coupled method due to the instability of quinoid 6(*R*)-dihydrobiopterin to side-chain rearrangement. Absorbance values, $A(t)$, vs time, t, acquired at 2 points/s were directly fitted using nonlinear regression to $A(t) = A_f - (A_f - A_o)e^{-k_{dehyd}t}$, where the initial absorbance, A_o, the final absorbance, A_f, and the decay constant, k_{dehyd}, were optimized parameters. At any given pH the variations in $A_f - A_o$ were within expected pipetting error.

The partitioning of decay of synthetic 4a-hydroxy-6(*R*)-tetrahydrobiopterin between dehydration and an alternate pathway at pH 8.4 was measured by HPLC in the presence of NADH and dihydropteridine reductase as above. The fate of chemically synthesized material was compared to that produced by phenylalanine hydroxylase[30] in the absence of a reducing system by chromatographic separation of the byproduct and quinoid dihydrobiopterin on Kromasil C_{18}.

Rate, k_{dehyd}, vs temperature, T, data were fitted by nonlinear regression to $k = (kT/h)e^{-\Delta H^*/RT}e^{\Delta S^*/R}$. Since the above absorbance vs time curves gave errors approximately proportional to k, the temperature curve was fitted with inversely proportioned weights. This gives results very close to the usual Arrhenius treatment.

Determination of pK_a Values. A 7.2 mM solution of divicine·$^1/_2H_2SO_4$ in 0.01 M HCl on ice was oxidized with an equal volume of 0.01 M Br_2 in H_2O and kept on ice. Aliquots were diluted 100-fold into various buffers already at 3.5 °C and the spectra acquired within 50 s. The basic pK was determined in 0.1 M sodium acetate buffers over the pH range 3.5−5.9 and in 0.01 M HCl. The acidic pK was ascertained in 0.1 M sodium phosphate and 0.1 M sodium borate buffers spanning pH 7.5−10.2 and in 0.01 M NaOH. The pK values of quinoid 6-methyl-6-phenyldihydropterin **8** (R_1 = Me, R_2 = Phe), 6-methyl-6-phenyltetrahydropterin, and 6,6-dimethyltetrahydropterin were determined at 25 °C in 0.01 ionic strength formate, acetate, and succinate buffers.



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Results

Spectra and Ionization of Quinoid Divicine and Derivatives. The spectra of the cationic, neutral, and anionic forms of quinoid divicine hydrate **9** are shown in Figure 1. The basic and acidic pK_a values for the transitions among these species at 3.5 °C were found to be 5.2 ± 0.05 and 8.8 ± 0.2, respectively. The uncertainty in the latter measurement is due to the instability of quinoid divicine in alkaline conditions. Rapid acidification of a solution having the spectrum of the full anion gave a curve nearly identical, though somewhat attenuated, to that of material freshly diluted into acid. The spectra of quinoid N^6-(2′(*S*)-aminopropyl)-, N^6-(2′(*S*)-aminopentyl)-, and N^6-(2′(*R*)-amino-3′(*R*),4′(*S*)-dihydroxypentyl)divicines **6** and **7** (R=Me, Pr, or dihydroxypropyl, respectively) in 0.1 N HCl are similar to that of the quinoid divicine hydrate cation except for an increase of about 7 nm in λ_{max} to 270 nm. A peak at 224 nm was observed with the aminopropyl analog, but only a shoulder

(30) Purified phenylalanine hydroxylase, 3 units/mL, was preincubated with 1 mM L-phenylalanine in 0.025 M Tris−HCl, pH $8.4_{10°C}$ at 27 °C for 5 min and cooled to 10 °C and the reaction initiated with tetrahydropterin (0.2 mM final concentration).

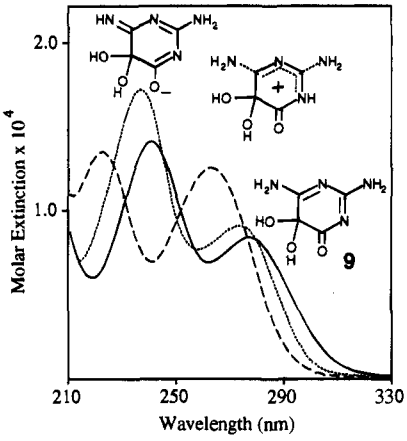


Figure 1. UV spectra of quinoid divicine hydrate **9** in 0.01 N HCl (- - -), 0.1 M sodium phosphate pH 7.0 (—), and 0.01 M NaOH (···). The tautomeric structure of **9** is not known.

was seen with the latter two compounds in this region. The basic pK_a values for 6-methyl-6-phenyltetrahydropterin, 6,6-dimethyltetrahydropterin, and quinoid 6-methyl-6-phenyldihydropterin were found to be 4.15 ± 0.07, 5.8 ± 0.1, and 5.1 ± 0.1, respectively.

Oxidation of Triaminopyrimidinone 4. The use of halogen only for the oxidation of N^6-substituted 2,5,6-triamino-4-pyrimidinones, such as **4**, requires an excess of reagent which has been ascribed to formation of covalent halogen adducts.[14] In the present work it was found that the subsequent hydrolysis and cyclization of such overoxidized material resulted in 4a-hydroxytetrahydropterins that were contaminated with byproducts observable by HPLC. The byproducts chromatographed later than the desired 4a-hydroxy-6-alkyltetrahydropterin, but had somewhat similar spectra, and could be largely converted to the corresponding 6-alkyltetrahydropterin by addition of reductant. It is proposed, therefore, that these byproducts may also be halogen adducts, possibly of the 4a-hydroxytetrahydropterin. The byproducts were found to interfere with the characterization of the 4a-hydroxy compounds, for example, by superimposition of their spectral decomposition patterns. The use of stoichiometric bromine with catalytic ferricyanide virtually eliminated the extraneous compounds from the chromatogram and the spectral artifacts. The omission of the ferricyanide (which was observed to be a more rapid oxidant than bromine) resulted in a mixture of not only the byproducts found with excess halogen but also unoxidized starting pyrimidine.

Cyclization to 4a-Hydroxytetrahydropterins. The rate of cyclization of quinoid N^6-(2′(*S*)-aminopropyl)divicines **6** and **7**, R = Me) to give **2** (R = Me) in borate buffer at 1 °C as a function of pH followed a bell-shaped curve with a maximum near pH 9.8 (Figure 2). Considering the observation of an anionic species of the related quinoid divicine hydrate **9** (see above), these rates were fitted to eq 1 which is based on Scheme 3.

Several provisional simplifying assumptions were made. Any shift with pH in the equilibrium between the anhydrous form shown and the predominant hydrate was neglected. Further, the cyclization of anion $\mathbf{6}^-$ was set to zero, since it is likely much slower than cyclization of the neutral species $\mathbf{6}^0$. Lastly, although pK_3 for dissociation of the zwitterion ($\mathbf{6}^{+/-}$) to the anion may be somewhat higher than pK_1, these were treated as equal (and similarly, pK_2 equal to pK_4) to give

$$k_{cyc} = k_5K_1/([H^+] + K_1 + K_2 + K_1K_2/[H^+]) \qquad (1)$$

The fit of the data to eq 1 has two solutions with pK values of

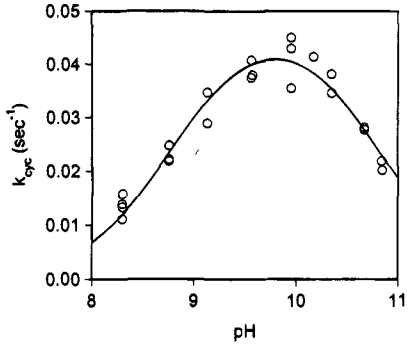
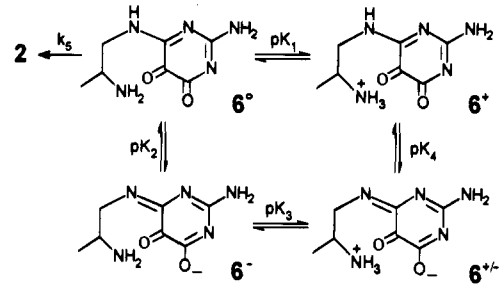


Figure 2. Rate of cyclization of **6** (R = Me) vs pH at 1 °C in 0.01 M sodium borate.

Scheme 3



about 8.8 and 10.8. The higher value is assigned to pK_1 for the ionization of the side-chain amino group (cf. 1,2-propanediamine first association constant, ≅10.5 at 3 °C), and the lower value to the formation of the pyrimidine anion (see above). In this view, at pH 9.8, where the observed rate is maximum, only 0.8% of **6** would be present as the reactive neutral species, giving a value for $k_5 = 4.8\ s^{-1}$. However, the calculated value of k_5 decreases somewhat if pK_3 is allowed to be greater than pK_1.

A bell-shaped curve was also observed for the rate of cyclization vs pH of quinoid N^6-(2′(*S*)-aminopropyl)divicine in MeOH at −20 °C (data not shown). The maximum rate of about $0.0015\ s^{-1}$ was found at pH 9.1.[28]

The overall yield of the conversion of 2,5,6-triamino-N^6-(2′-(*S*)-aminopropyl)-4-pyrimidinone **4** (R = Me) to **2** (R = Me), i.e., of oxidation, hydrolysis, and cyclization, in MeOH was measured by subsequent enzyme-catalyzed dehydration and reduction of **2** to 6(*S*)-methyltetrahydropterin **1** (R = Me). The NADH consumed upon completion of the reaction was stoichiometric with starting **4**, and the amount of **1** (R = Me) found by HPLC was 97.± 3%. In the standard protocol (6 min at 0 °C) greater than 98% of the quinoid divicine derivatives **6** and **7** (R = Me or Pr) cyclizes while only about 4% of the corresponding quinoid 6-alkyldihydropterin **3** (R = Me or Pr) is produced.

Formation of 7-Substituted Pterins. Cyclization of quinoid N^6-(2′-substituted 2′-aminoethyl)divicines **6** and **7** which are free of any 1′-substituted impurity produced 4a-carbinolamines which upon dehydration contained 7-substituted **12** as well as the expected 6-substituted quinoid dihydropterins. These 7-substituted compounds were characterized by comparison of their UV spectra, electrochemical voltamograms, and HPLC retention to authentic standards. The 6- and 7-isomers of the quinoid methyl- and propyldihydropterin products are sufficiently stable and resolved by reversed-phase HPLC that they were directly quantitated. On the other hand, the isomer ratio produced from the 1′(*R*),2′(*S*)-dihydroxypropyl compound was best determined by further reduction to 6- and 7-tetrahydrobiopterin by dihydropteridine reductase and NADH before chromatographic separation.

Cyclization of **6** and **7** (R = Me) in MeOH at 0 °C at pH 9.1[28] gave a ratio of quinoid 6- to quinoid 7-methyldihydropterin of 24:1. Only little sensitivity to temperature was seen; a ratio of 28:1 was found at 37 °C in the same solvent. A 1:1 mixture of water and MeOH at 0 °C and pH 9.1[28] produced almost the same result as in pure MeOH. However, partitioning to the 7-isomer **12** (R = Me) increased substantially in water (6.5:1 at 0 °C; 7.5:1 at 27 °C, and 8:1 at 37 °C, 25 mM Tris–HCl, pH 8.4, at all temperatures). At pH 7.4, although the overall cyclization yield decreased,[31] little change in partitioning was observed (7:1 at 37 °C). Cyclization in the lower dielectric constant medium *p*-dioxane/MeOH (9:1) (pH 9.1)[28] decreased the content of **12** (R = Me) to a ratio of 65:1, although several additional byproducts were also generated.

Cyclization of **6** and **7** (R = Pr) in MeOH at 0 °C gave the same ratio of quinoid 6-propyl- to quinoid 7-propyldihydropterin as the methyl analog. However, much higher amounts of 7-substituted isomer were formed from **6** and **7** (R = 1′(*R*),2′(*S*)-dihydroxypropyl); standard cyclization in MeOH at 0 °C produced (after reduction by dihydropteridine reductase and NADH) a ratio of 6- to 7-tetrahydrobiopterin of 15:1. In aqueous 25 mM Tris–HCl, pH 8.4 at 0 °C and pH 7.4 at 37 °C, ratios of 3:1 and 4:1, respectively, were observed.

Chromatograms of 4a-hydroxytetrahydropterins **2** immediately after completion of cyclization showed an unresolved shoulder on the tailing edge of the peak of the main product. The spectrum of this shoulder was similar to that of the main compound, but with a somewhat lower ratio of 247 to 290 nm absorbance. This minor component disappeared faster than the main component in concert with formation of the related quinoid 7-substituted dihydropterin **12**. If the minor component associated with **2** (R = Me) was isolated and reinjected, a greatly enhanced abundance of **12** (R = Me) was produced. Thus, this shoulder is likely either 4a-hydroxy-7-methyltetrahydropterin **11** (R = Me) or its precursor. The shoulder was found to be absent from **2** (R = Me) generated from 6(*S*)-methyltetrahydropterin by a phenylalanine hydroxylase reaction in the absence of a quinoid dihydropterin reductant.[32] Further, upon dehydration no quinoid **12** (R = Me) was detected (limit of sensitivity about 0.2%) as a result of the enzymatic reaction. Therefore, the shoulder is not likely to be a chromatographic artifact, nor is it derived from **2** (R = Me) itself under the conditions of this experiment.

Dehydration of 4a-Hydroxytetrahydropterins. The effect of ionic strength on the dehydration of **2** (R = Me) in 25 mM Tris–HCl, pH 7.4, at 10 °C was examined at varying concentrations of KCl. As the concentration was increased to 0.5 M KCl, the rate of dehydration increased nonlinearly from 0.0055 s^{-1} with buffer only to 0.007 s^{-1}. From 0.5 M KCl to 2.0 M no further significant change of rate was found. Considering the minor impact of salt, studies of the influence of buffer type, concentration, and pH on the rate of dehydration were performed without maintaining constant ionic strength.

The dehydration of **2** (R = Me) was monitored in various buffers at 17 °C at the isosbestic wavelength for the conversion of **3** (R = Me) to 6-methyl-7,8-dihydropterin. Progress curves followed a first-order decay over at least 3–4 half-lives.[16] Upon completion of dehydration, analysis of the reaction by HPLC with detection at 340 nm confirmed that the only products absorbing at this wavelength were **3** (R = Me), 6-methyl-7,8-dihydropterin, and a small peak of fully oxidized 6-methylpterin. Measurement of the dehydration of **2** (R = Me) at 10 °C in 0.025 M Tris–HCl, pH 8.4, by following its disappearance by HPLC gave a rate constant identical to that found spectrophotometrically.

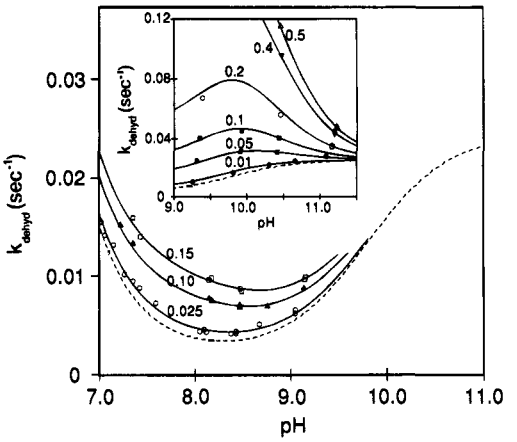


Figure 3. Rate of dehydration of **2** (R = Me) at 17 °C vs pH in Tris–HCl buffers (main graph) and Me_3N–HCl buffers (inset). Buffer molarity is indicated above each curve. Comparable data for 0.05 M Tris–HCl have been omitted for clarity. The dashed line which is common to both the Tris and Me_3N data represents the rate calculated by eq 2 in the absence of buffer.

When **2** (R = Me) is generated *in situ* by dilution of **6** and **7** (R = Me) into alkaline buffers, a similar progress curve is observed except for the early time points before cyclization is complete. Since cyclization slows considerably at pH values outside of the optimal region (see above), and in contrast to dehydration appears to be only weakly catalyzed by buffer,[33] preformed 4a-carbinolamines were used for all experiments.

The yield of dehydration of **2** (R = Me) at 17 °C in Tris–HCl and Me_3N–HCl buffers appears to be nearly quantitative from pH 7 to pH 11.3. This was determined by chromatographic analysis of the 6-methyltetrahydropterin and 6-methyl-7,8-dihydropterin produced by addition of 2-mercaptoethanol to a reaction after spectrophotometric monitoring indicated completion of dehydration. The yield was not significantly affected by increasing buffer concentration up to 0.4 M, although the product ratio shifts to favor 6-methyl-7,8-dihydropterin due to buffer catalysis of quinoid dihydropterin tautomerization.[34]

In Tris–HCl, rates of dehydration were linear with buffer concentration from pH 7 to pH 9.2 over the studied range (0.025–0.15 M) (Figure 3). At pH 7.3 and 17 °C, reactions were observed to be linear with buffer concentration up to 1 M (data not shown). This was also the case with the other amine buffers investigated, including Me_3N–HCl which was used to determine dehydration rates between pH 9.2 and pH 11.3. The Tris–HCl and Me_3N–HCl data at 17 °C were simultaneously fitted to eq 2, where k_o is the first-order rate of dehydration of

$$k_{dehyd} = k_o f_o + k_H[H^+] + k_{OH^-}[OH^-]f_o + k_{Tris}[Tris]f_o + k_{Tris-H^+}[Tris{-}H^+] + k_{Me_3N}[Me_3N]f_o + k_{Me_3NH^+}[Me_3NH^+] \quad (2)$$

the neutral fraction (f_o) of **2** (R = Me), k_H and k_{OH^-} are the rate constants for proton and hydroxide catalysis, and k_{Tris}, k_{Me_3N}, k_{Tris-H^+}, and $k_{Me_3NH^+}$ are the second-order rate constants for catalysis by Tris base and trimethylamine base and their respective hydrochloride salts. In this model, acid catalysis of dehydration was assumed for simplicity to apply equally to both

(31) Pike, D. C.; Hora, M. T.; Bailey, S. W.; Ayling, J. E. *Biochemistry* **1986**, *25*, 4762–4771.
(32) As in ref 30 except phenylalanine hydroxylase, 0.25 unit/mL.
(33) Bailey, S. W.; et al. Unpublished result.
(34) Archer, M. C.; Scrimgeour, K. G. *Can. J. Biochem.* **1970**, *48*, 278–287.

Table 1. Catalytic Constants for Buffer Catalysis of Dehydration of **2** (R = Me) at 17 °C

buffer	charge or pH	rate constant (M^{-1} s^{-1})
potassium phosphate	7.40	0.35[a]
potassium phosphate	8.0	0.19[a,c]
HEPES–Na	8.40	0.08[a]
$(HOC_2H_4)_3N$–HCl	8.35	0.05[a]
Tris–HCl	cation, k_{Tris-H^+}	0.053[b]
Tris–HCl	8.40	0.036[b]
Tris	base, k_{Tris}	0.023[b]
sodium borate	8.76	0.06[d]
sodium borate	9.60	0.17[d,e]
Me_3N–HCl	cation, $k_{Me_3N-H^+}$	0.20[b]
Me_3N	base, k_{Me_3N}	1.0[b]

[a] Determined over the range of 5–200 mM. [b] Calculated from the data of Figure 3 using eq 2. [c] Determined at 25 °C. [d] From the data of Figure 4. [e] Nonlinear; extrapolated to infinite borate dilution.

the neutral and anionic species of **2**, the pK for which was included as a calculated parameter in the equation.

The rate of dehydration of **2** (R = Me) at 17 °C extrapolated to zero buffer as a function of pH is shown as the dashed line in Figure 3 and inset. The minimum rate of dehydration in the absence of buffer occurs at about pH 8.25 with a value calculated by eq 2 of 0.0035 s^{-1} ($t_{1/2}$ = 200 s) at 17 °C. However, the rate of spontaneous dehydration of the neutral carbinolamine (k_o) at this temperature was calculated to be 0.002 s^{-1}. Therefore, at the pH of maximum stability in the absence of buffer about 60% of the dehydration is due to an uncatalyzed (except by solvent) reaction. The remainder is divided between the proton- and hydroxide-promoted reactions with $k_H = 1.3 \times 10^5$ M^{-1} s^{-1} and k_{OH^-} = 400 $M^{-1}s^{-1}$, respectively. The increase in the buffer-independent rate with increasing pH is consistent with an ionization having a pK = 9.8.

In the presence of buffer the pH at which the rate of dehydration is minimum depends on the buffer type and concentration. Of those examined, dehydration was catalyzed the least by Tris in the region of maximum intrinsic carbinolamine stability. Dehydration promoted by the hydrochloride salt of Tris was found to be more than 2 times as effective as by Tris base (Figure 3 and Table 1). As a result, the pH of maximum stability to dehydration increases with increasing Tris concentration, for example, from pH 8.3 in 0.025 M Tris to pH 8.7 with 0.15 M Tris. The values of k_{Tris}, k_{Tris-H^+}, k_{Me_3N}, and $k_{Me_3NH^+}$ for dehydration of **2** (R = Me) as well as catalytic constants for other buffers at selected pH values are listed in Table 1. In Tris–HCl, pH 8.4, at 17 °C the dehydration rate of **2** (R = Pr) extrapolated to zero buffer and the catalytic constant for Tris at this pH were found to be identical within experimental error to those for the methyl analog. At pH 8.76 catalysis of dehydration of **2** (R = Me) by sodium borate was nearly linear over the measured range of 0.025–0.15 M. However, a nonlinear relationship of rate versus borate concentration was observed in the pH range 9.3–9.85, illustrated in Figure 4. The sharp drop in rates in Me_3N–HCl above pH 10 (Figure 3, inset) indicates little if any catalysis of dehydration by buffer base above pH 12.

The rates of dehydration of 6(*S*)-methyl- and 6(*S*)-propyl-4a-hydroxytetrahydropterin were measured as a function of temperature between 3 and 37 °C in 0.025 M Tris–HCl at pH 7.4 and 8.4. The parameters listed in Table 2 show that at pH 7.4 and 8.4 the methyl and propyl analogs have the same properties.[35] The results obtained by directly measuring quinoid dihydropterin formation at the wavelength isosbestic with 7,8-dihydropterin at 340–350 nm or by coupling the reaction to NADH and dihydropteridine reductase were the same within experimental error. At pH 7.4 the rate constants for the two 6-alkyl analogs at 37 °C are near 0.040 s^{-1} ($t_{1/2}$ = 17 s). The dehydration rate of **2** (R = Me) generated by the action of phenylalanine hydroxylase on 6(*S*)-methyltetrahydropterin[32] was examined in 0.025 M Tris–HCl, pH 8.4, at 10 °C and was found to be identical to that of the synthetic material.

(35) An activation energy for dehydration of enzymatically generated 4a-hydroxy-6(*R*,*S*)-methyltetrahydropterin in 0.02 M Tris–Cl, pH 8.5, as determined at 244 nm has previously been reported.[6]

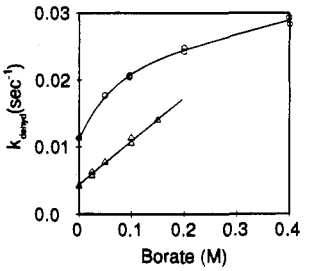


Figure 4. Rate of dehydration of **2** (R = Me) at 17 °C in sodium borate, pH 8.7 (Δ) and pH 9.6 (○), vs buffer concentration. The solid markers are the rates of dehydration in the absence of buffer at pH 8.7 and 9.6 calculated from the data of Figure 3 using eq 2.

Table 2. Activation Parameters[a] for the Dehydration of **2** (R = Me and Pr)

	ΔH^* (kcal/mol)	ΔS^* ((cal/mol)/K)	ΔG^* [b] (kcal/mol)
pH 7.4			
Me	12.065	−26.155	20.173
Pr	12.061	−26.015	20.126
pH 8.4			
Me	15.050	−17.468	20.465
Pr	14.852	−18.075	20.455

[a] Data collected using the assay coupled to NADH consumption. [b] Calculated for 37 °C.

The measurement of the dehydration rate of 4a-hydroxy-6(*R*)-tetrahydrobiopterin **2** (R = 1′(*R*),2′(*S*)-dihydroxypropyl) is complicated by the formation of another product thought to be a side-chain 4a-cyclic adduct of tetrahydrobiopterin (e.g., **10**). Spectrophotometric rate constants for dehydration of 4a-hydroxy-6(*R*)-tetrahydrobiopterin acquired over less than 1 half-life in the presence of dihydropteridine reductase and NADH appear to be similar to those of the methyl and propyl analogs (see above). However, monitoring the fate of 4a-hydroxy-6(*R*)-tetrahydrobiopterin by HPLC at 10 °C in 0.025 M Tris–HCl pH 8.4, and analysis of the data according to the kinetic pattern shown in Figure 5, gave a rate constant for dehydration (k_1) of 0.0018 s^{-1}, about 85% of that of the alkyl analogs. This is consistent with the rate observed by HPLC for the overall decay at pH 7.5 and 23 °C of 4a-hydroxy-6(*R*)-tetrahydrobiopterin produced by tyrosine hydroxylase[12] which is about 80% of that found in the current study for **2** (R = Me) under similar conditions.

Solvent Kinetic Isotope Effects. An isotope effect of D_2O vs H_2O of 2.61 ± 0.07, 2.23 ± 0.15, and 3.49 ± 0.08 was found for dehydration at 17 °C in 10 mM Tris–HCl, pH 7.4, 10 mM Tris–HCl, pH 8.2, and 10 mM Me_3N–HCl, pH 10.9, where the reaction is primarily due to the proton-catalyzed reaction, spontaneous loss of hydroxide from the neutral species, and dehydration at a pH where the anionic species predominates, respectively.

An Alternate Product from 4a-Hydroxy-6(*R*)-tetrahydrobiopterin. When a solution of 4a-hydroxy-6(*R*)-tetrahydrobiopterin is diluted into aqueous buffer, a second peak in addition to the expected quinoid 6(*R*)-dihydrobiopterin **3** (R = 1′(*R*),2′(*S*)-dihydroxypropyl) is detected by HPLC. Although not identified directly, the following observations indicate that

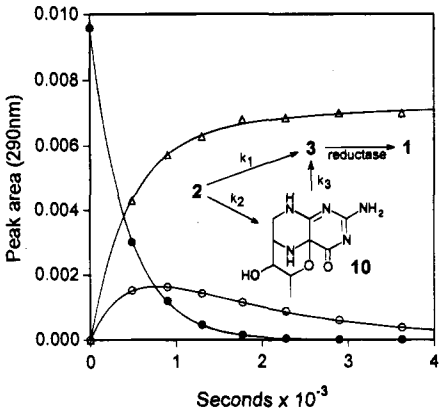


Figure 5. Decay of 4a-hydroxytetrahydrobiopterin **2** (R = 1′(*R*),2′(*S*)-dihydroxypropyl) (●) in 0.025 M Tris−HCl, pH 8.4, at 10 °C in the presence of NADH and dihydropteridine reductase to **3** (detected as 6(*R*)-tetrahydrobiopterin **1** (Δ)) and another product tentatively identified as a side-chain 4a-cyclic adduct of tetrahydrobiopterin (e.g., **10**) (○) monitored by HPLC.

this new compound is a 4a-adduct of a side-chain hydroxyl group: (i) This byproduct, which elutes slightly earlier than quinoid 6(*R*)-dihydrobiopterin on the reversed-phase system used for analysis of carbinolamines, has a UV spectrum (λ_{max} = 247 and 291 nm, $\epsilon_{247}/\epsilon_{291}$ = 2.45, pH 8.2) very similar to that of **2**, and has no electrochemical activity between −0.2 and +0.6 V vs Ag/AgCl. (ii) When isolated by HPLC, it slowly decays to a compound with the UV, electrochemical, and chromatographic properties of quinoid 6(*R*)-dihydrobiopterin. (iii) Decomposition of similarly isolated material in the presence of NADH and dihydropteridine reductase gives exclusively 6(*R*)-tetrahydrobiopterin. (iv) No related compound can be detected during the spontaneous dehydration of **2** (R = Me or Pr).

A very small chromatographic peak with UV spectrum and retention time corresponding to the above byproduct can also be detected following oxidation of 6(*R*)-tetrahydrobiopterin to quinoid 6(*R*)-dihydrobiopterin (e.g., by bromine in unbuffered water). However, the amount of this material produced during dehydration of 4a-hydroxytetrahydrobiopterin and the kinetics of its appearance even in the presence of NADH and dihydropteridine reductase (Figure 5) show that it is generated primarily in parallel with, rather than from, quinoid 6(*R*)-dihydrobiopterin. The decomposition of the byproduct to quinoid 6(*R*)-dihydrobiopterin (k_3) is considerably slower than dehydration (k_1). For example, in 0.025 M Tris−HCl, pH 8.4, at 10 °C, k_3 (6×10^{-4} s^{-1}) was about one-third that of the dehydration of 4a-hydroxytetrahydrobiopterin (k_1). Dehydration (k_1) predominated formation (k_2) of **10** by a factor of about 3 (assuming the cyclic adduct has an extinction coefficient similar to that of the starting material). In 0.04 M KPO_4, pH 8.0, at 25 °C, k_2 was found to be almost 10-fold slower than k_1. 4a-Hydroxy-6(*R*)-tetrahydrobiopterin generated by phenylalanine hydroxylase also produced this byproduct. The ratio of byproduct to quinoid dihydrobiopterin observed at various times after completion of the enzymatic consumption of 6(*R*)-tetrahydrobiopterin exactly corresponded to those found from chemically synthesized 4a-hydroxytetrahydrobiopterin allowed to decay in the absence of NADH and dihydropteridine reductase. The maximum yields of byproduct from enzymatically and chemically generated material were the same within experimental error.

Discussion

Hydration and Ionization of Quinoid Divicine and Derivatives. Spectrophotometric studies suggest formation of an anion of quinoid divicine **9** (which is more stable than its N^6-derivatives) with an apparent pK_a of about 8.8. That quinoid divicine and its derivatives **6** and **7** are primarily hydrates of the 5-oxo group in aqueous solution (as in the case of alloxan) can be seen from the similarity of the spectra of their neutral species to those of 4a-hydroxytetrahydropterins. Since the effects of ionization and solvent type on the extent of hydration are not currently understood, the observed p*K* values may be a composite of several species. Nonetheless, formation of such an anion correlates well with the effect of pH on cyclization to, and dehydration of, 4a-hydroxytetrahydropterins. That **6** and **7** (R = Me) is primarily the hydrate is also demonstrated by FAB mass spectrometry. Although a minor component of the anhydrous species **6** was also detected, it is not clear how much of this may have been formed during sample volatilization/ionization from the probe.[36]

The ease with which quinoid divicine hydrate and related N^6-derivatives are formed by mild acidic hydrolysis of pyrimidine 5-imines is in contrast to the behavior of dehydrouramil (the 5-imino analog of alloxan). This latter compound has been reported to be hydrolyzed to an acid-stable 5-carbinolamine.[37] It is interesting that substitution of the 2- and 6-amino groups for the oxygen functionality should have such a marked stabilizing effect. On the other hand, these researchers reported difficulty in locating two of the three hydrogens on the putative 5-amino group in their crystallographic data. Further, the bond lengths between C5 and the attached nitrogen and oxygen were identical (1.389 Å). Therefore, the possibility that the product of dehydrouramil hydrolysis may instead have been the ammonium chloride salt of alloxan hydrate, which has a nearly identical crystallographic structure,[38] should be considered.

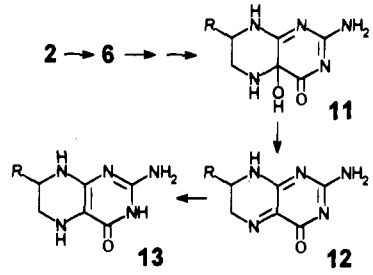
The observed pK_a of 5.2 is assigned to protonation of the extended guanidinium system of **9**. A similar protonation to form the monocation of quinoid 6,7-dimethyldihydropterin has been deduced by ^{15}N NMR.[39] This latter finding was further confirmed in the present study by the similarity of the dissociation constants of quinoid 6,6-dimethyldihydropterin **8** (R_1, R_2 = Me)[23] and the 6-methyl-6-phenyl analog **8** (R_1 = Me, R_2 = Phe). This is in contrast to the decrease in p*K* of 1.65 which a phenyl group induced in their respective tetrahydropterins, consistent with earlier studies showing that the monocation of tetrahydropterins is produced by protonation of N5.[39-41]

Synthesis of 4a-Hydroxytetrahydropterins. The production of 4a-hydroxytetrahydropterins in high yield that are suitable without purification for chemical characterization and use as dehydratase substrates was found to be subject to a few precautions: (i) The starting 5-aminopyrimidine **4** should be free not only of other pyrimidine contaminants, but also any general acids or bases that can act as dehydration catalysts (e.g., Et_3N). (ii) Oxidation of **4** with stoichiometric bromine and catalytic ferricyanide avoids impurities which are produced if bromine only is used. (iii) The solvent should contain less than 50% water in order to minimize formation of the 7-isomer **11**. (iv) Upon completion of hydrolysis of the 5-iminopyrimidine **5**, cooling the resulting quinoid N^6-(2′-aminoalkyl)divicines **6** and **7** in a dry-ice bath permits titration to the optimal pH for cyclization without premature unfavorable reaction (see below).

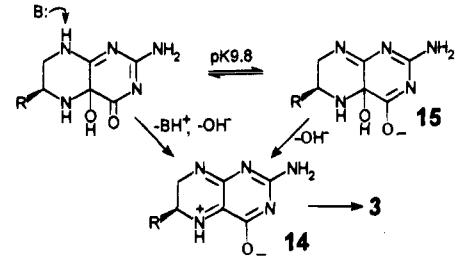
(36) A very low response to electrochemical reduction of **6** and **7** during HPLC analysis in comparison to quinoid dihydropterins **3** further suggests an equilibrium considerably favoring hydration.[33]

(37) Poje, M.; Ročić, B.; Sikirica, M.; Vicković, I.; Bruvo, M. *J. Med. Chem.* **1983**, *26*, 861−864.
(38) Mootz, D.; Jeffrey, G. A. *Acta Crystallogr.* **1965**, *19*, 717−725.
(39) Benkovic, S. J.; Sammons, D.; Armarego, W. L. F.; Waring, P.; Inners, R. *J. Am. Chem. Soc.* **1985**, *107*, 3706−3712.
(40) Kallen, R. G.; Jencks, W. P. *J. Biol. Chem.* **1966**, *241*, 5845−5850.
(41) Schwotzer, W.; Bieri, J. H.; Viscontini, M.; von Philipsborn, W. *Helv. Chim. Acta* **1978**, *61*, 2108−2115.

Scheme 4



Scheme 5



The yield of 4a-hydroxytetrahydropterin is governed primarily by the competition of the rate of attack of the exocyclic 2'-amino group on the 5-carbonyl with the rate of degradation of the unstable pyrimidine ring of quinoid divicine. This decomposition rate has been examined with the almost noncyclizable quinoid N^6-(3'-aminobutyl)divicine which disappears with a first-order rate constant of 0.043 s^{-1} in water at pH 7.4 and 27 °C.[31] Thus, the increasing yield of tetrahydropteridines previously reported with increasing pH[14,31] is directly related to the rate of condensation of **6** and **7**. However, an unexpected decrease in the rate of cyclization was observed in more alkaline conditions (Figure 2), which is consistent with an ionizable group with a p$K \simeq 8.8$, similar to the spectrally observable pK for formation of the anion of quinoid divicine hydrate **9**. This suggests that the charge, which likely resides primarily on O^4, inhibits nucleophilic attack on C5.

Conditions which are optimal for a high yield of 4a-hydroxytetrahydropterins are generally appropriate for C6-stereospecific synthesis of tetrahydropterins. In this case, rapid dehydration of the 4a-carbinolamine is desired, but must be catalyzed in a manner that does not promote tautomerization of the resulting quinoid dihydropterin to the 7,8-dihydropterin. A slight shift to a neutral or slightly acidic environment is sufficient for this purpose considering the effectiveness of proton-catalyzed dehydration. After reduction and crystallization[14] the 7-alkyltetrahydropterin contamination is less than the limit of detection (0.1%).

Formation of 7-Substituted Pterins. The end product of tetrahydrobiopterin biosynthesis places a dihydroxypropyl group at the 6-position. However, 7-tetrahydrobiopterin **13** (R = 1'(*R*),2'(*S*)-dihydroxypropyl), which is an inhibitor of phenylalanine hydroxylase,[42,43] is produced in trace quantities by normal individuals and in significant amounts by children born with a mild form of hyperphenylalaninemia. Since these cases do not appear to be deficient in the other enzymes involved in phenylalanine metabolism, 4a-hydroxytetrahydropterin dehydratase has been examined for defects.[44] It has been suggested that 7-tetrahydrobiopterin is formed by ring-opening of 4a-hydroxytetrahydrobiopterin (**2** → **6**), followed by an intramolecular rearrangement via a spiro compound to give a quinoid N^6-(1'-substituted aminoethyl)divicine. This then recondenses to 4a-hydroxy-7-tetrahydrobiopterin **11** (R = 1'(*R*),2'(*S*)-dihydroxypropyl) which ultimately leads to the observed products (Scheme 4).[45,46]

The results presented here demonstrate that the ring-opened quinoid divicines **6** and **7** can give rise to 7-substituted pteridines, although the intermediates are yet to be elucidated. The extent of 7-isomer formation is only slightly influenced by pH or temperature, but is significantly affected by solvent. For the purpose of generating high-purity 6-substituted 4a-hydroxytetrahydropterins, this alternate pathway can be effectively suppressed in methanol or other low dielectric solvents. On the other hand, considerable 7-substituted product is formed by cyclizations carried out in totally aqueous solution, especially with **6** and **7** (R = dihydroxypropyl) which gives about 20% 7-tetrahydrobiopterin under physiological conditions (37 °C, pH 7.4). These observations, therefore, confirm the last half of the proposed pathway.

Dehydration Kinetics. The thermodynamic parameters for dehydration of the methyl and propyl derivatives of **2** are within experimental error identical to each other at pH 7.4 or 8.4 (near the point of optimal stability). Although part of the total rate of disappearance of 4a-hydroxy-6(*R*)-tetrahydrobiopterin is due to formation of a second product (see below), the dehydration component appears to be only slightly slower than that of the 6-alkyl analogs. On the other hand, dehydration rates are substantially faster if there is more than one methyl substituent at the 6- and/or 7-positions.[33]

The condensation of a single isomer of **6** and **7** can generate two 4a-epimers, with the possibility of asymmetric induction by the existing chiral center. The maintenance of rate constant over many half-lives of dehydration indicates that either the properties of the epimers are very similar, and/or that one is preferentially formed during cyclization. In this context it is interesting to note that the dehydration rate of synthetic **2** (R = Me) is the same as that produced by phenylalanine hydroxylase from 6(*S*)-methyltetrahydropterin.[47]

Catalysis of dehydration of 4a-hydroxytetrahydropterins by proton and the acid form of buffer is readily understood in view of previous studies of carbinolamine dehydration.[15] On the other hand, the rate of dehydration extrapolated to zero buffer increases above pH 8.2 in a manner suggesting involvement of an ionizable group with p$K = 9.8$ (Figure 3) (cf. quinoid divicine hydrate **9** (pK = 8.8). Further, significant general base catalysis was observed, although only below pH 11.5. This is unexpected considering that base-catalyzed dehydration is usually found only with carbinolamines formed from the condensation of a carbonyl group with a weak base such as semicarbazones or oximes.[15,48] Equation 2 includes catalysis by hydroxide acting on the neutral but not the anionic species of the carbinolamine. However, this is kinetically indistinguishable from an equation in which the $k_{OH^-}[OH^-]f_0$ term is replaced by $k_{an}f_{an}$, where k_{an} is the rate of dehydration of the anionic fraction (f_{an}) (Scheme 5). Interaction of base catalyst with a proton, presumably other than that on the fairly basic N5, in a manner concerted with hydroxide cleavage might alleviate some of the unfavorable energy involved in separation of oppositely charged products

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to give the zwitterion **14**. This mechanism would be defeated once the full anion **15**$^-$ is formed. It will be of interest to determine whether base catalysis is also utilized by the 4a-hydroxytetrahydropterin dehydratase.

Catalysis of dehydration by borate (which ionizes primarily by association with OH^-) is linear with buffer concentration at pH 8.8, but is nonlinear at pH 9.6 in a manner similar to the change in the ^{11}B chemical shift with increasing alkali-metal tetraborate.[49] As the pH of concentrated borate buffers is increased above neutral the total polyborates have been shown to decrease, and by pH 10 primarily $B(OH)_4^-$ and tetraborate are present.[50] The higher catalytic constant at low borate concentration at pH 9.6 than at pH 8.76, therefore, may be due to a different degree of polymerization. The high catalytic activity of phosphate monoanion (Table 1) is useful in the spectral assay of purified phenylalanine hydroxylase where buffer can act in lieu of 4a-hydroxytetrahydropterin dehydratase to temporally couple cofactor consumption to NADH oxidation by dihydropteridine reductase.

An alternate product of 4a-hydroxy-6(*R*)-tetrahydrobiopterin rearrangement, tentatively assigned to be an intramolecular 4a-adduct of $O^{1'}$ or $O^{2'}$ (the latter is arbitrarily illustrated as **10**) was observed to compete with dehydration.[51] That this phenomenon is not due to the decay of an undetected impurity in the synthetic preparation of **2** (R = dihydroxypropyl) was shown by the equivalent result using material generated by phenylalanine hydroxylase. In addition to the observations presented in the Results, the finding that conversion of the byproduct to quinoid 6(*R*)-dihydrobiopterin is catalyzed by the dehydratase[24] also argues for a structure closely related to that of a 4a-hydroxytetrahydropterin.

The solvent kinetic isotope effect ($k_H/k_D = 2.2$) on dehydration of **2** (R = Me) at pH 8.2 (in sufficiently dilute buffer that the water-catalyzed reaction (k_o) is dominant) was found to be close to that for the hydrolysis of benzhydrylidenedimethylammonium ion in the region where the latter reaction is pH-independent.[52,53] Considering that the fractionation factors[54,55] for the respective carbinolamine and imine reactants would be close to 1.0, the similarity suggests that the transition states for the two reactions probably resemble each other. Proton inventory experiments of the hydrolysis reaction indicate the involvement of several waters, possibly in a cyclic arrangement, in the transition state.[53] At pH 7.4, where about half of the dehydration is due to proton catalysis, k_H/k_D slightly increases and ΔS^* decreases by 8 (cal/mol)/K in comparison to that for the reaction at pH 8.2. These observations are consistent with a protonation of the hydroxyl that is concerted with formation of the transition state,[15,48,56,57] rather than in a preequilibration step (which would produce a lower, if not inverse, effect). In more alkaline conditions, where the majority of **2** (R = Me) is present as a monoanion, a considerably higher $k_H/k_D = 3.5$ was found. The negative charge may alter the network of water molecules involved in the transition state in comparison to that for the species present at pH 8.2. Alternatively, the 59% increase in k_H/k_D may reflect participation of an additional concerted proton abstraction by a base catalyst. Interestingly, this higher value associated with the alkaline reaction is close to that reported for buffer-independent dehydration of the carbinolamine formed from the rearrangement of α-amino-β-carboxymuconate ε-semialdehyde which yields quinolinate on loss of water.[58]

Conclusions

The results presented demonstrate that 4a-hydroxytetrahydropterins **2** can be prepared via intramolecular Schiff base condensation of derivatives of quinoid divicine **6**. The dehydration of **2** is subject to proton and general acid catalysis typical of other carbinolamines. However, general base catalysis was also observed which is largely defeated above pH 11.5 where formation of the anion of **2** is complete. This reaction, which would not be expected for the carbinolamine product of a condensation of a strongly alkaline amine such as in **6**, may be due to a concerted interaction of base with a pteridine proton other than on N5. The rate of dehydration at pH 7.4 and 37 °C (0.04 s^{-1}) was found to be too slow to allow for efficient cofactor regeneration during the *in vivo* phenylalanine hydroxylase reaction.[16] The availability of synthetic 4a-hydroxytetrahydropterins has enabled the first kinetic characterization of the dehydratase. The activity of this enzyme in the liver has recently been shown to be sufficient to catalyze dehydration at a rate that does not limit phenylalanine metabolism.[24] The mechanisms of nonenzymatic dehydration of carbinolamine **2** discussed in this paper provide a basis for understanding the dehydratase, which contains no metal or other cofactor.

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Supporting Information Available: Text describing the method for synthesis of **4** (R = Me) and the spectral characterization of this compound and its precursors (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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