Studies on 6-Methyl-5-deazatetrahydropterin and Its 4a Adducts[1]

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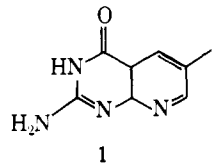
Abstract: The oxidation properties of 6-methyl-5-deazatetrahydropterin (**2**) have been examined. The compound (**2**) reacts with a variety of electrophilic reagents (including *N*-halosuccinimide, bromine, peroxy acid, and singlet oxygen) to generate the corresponding 4a adduct. These adducts (**3–6**) are characterized by their spectral properties (^{1}H and ^{13}C NMR, UV) and analytical data. The aqueous-solution chemistry of the adducts is a function of the nature of the 4a substituent. The bromo and chloro adducts are readily reduced (and the former compound is an active brominating agent), while the hydroxy adduct undergoes a facile decomposition with rupture of the pyrimidine ring. The compound **2** is a competitive inhibitor of both phenylalanine and tyrosine hydroxylases, whereas the 4a adducts (**3–6**) show no apparent interaction with the enzyme.

Introduction

Although there have been many studies on the pterin-dependent amino acid hydroxylases, the precise mechanism of the hydroxylation they effect is not well understood. It is often assumed that the hydroxylation proceeds by way of a reactive intermediate derived from the interaction of oxygen with the tetrahydropterin cofactor. Various structures have been proposed for this intermediate;[2-6] most take the form of, or are derived from, a 4a (or 8a) adduct of the tetrahydropterin. However, since there is no reported isolation or synthesis of such adducts (presumably due to a predisposition of these compounds to undergo rearrangement and/or elimination),[5-6] their properties are unknown and their intermediacy in the enzyme reaction remains unproved. Employing 6-methyl-5-deazatetrahydropterin (**2**), we have been able to isolate and characterize various 4a adducts and explore their chemistry and biochemistry, which is the topic of this report.

Results and Discussion

6-Methyl-5-deazatetrahydropterin: Synthesis and Properties. 6-Methyl-5-deazatetrahydropterin (**2**) as the monotrifluoroacetate salt is readily obtained in quantitative yield by hydrogenating the fully aromatic precursor (**1**)[7] in trifluoroacetic acid by using platinum dioxide catalyst. The use of trifluoroacetic acid as solvent for the reaction is dictated by the relative insolubility of both **1** and **2** in alternate media. The



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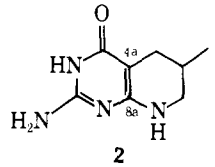

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excellent yield obtained under these conditions contrasts with the poor yields observed when similar compounds are reduced in aqueous acid.[8,9]

The structure of **2** is supported by spectral and analytical data (the ^{13}C NMR of **2** is shown in Figure 1, the ^{1}H NMR in Table I and Figure 2, and the UV at pH 1 and 8 in Figure 4). The assignment of the ^{13}C NMR resonances is straightforward and is based on the chemical shifts and multiplicities observed in the gated decoupled spectrum (see Experimental Section). The 360-MHz ^{1}H NMR is not a first-order spectrum due to the near coincidence of the resonances due to the H-5ax and H-6. It is possible, however, to estimate coupling constants with the aid of a computer simulation.[10] The magnitudes of $J_{5(7)a6}$ and $J_{5(7)e6}$ (Table I) accord with the values of axial-axial and equatorial-axial coupling constants seen in piperidine, tetrahydroquinoline, and tetrahydroquinazoline derivatives[11] indicating that **2** like the latter compounds adopts a rigid (on the NMR time scale) pseudo-chair conformation[11,12] with the methyl group equatorial.

The reduction of **1** with deuterium in trifluoroacetic acid-*d*[13] affords a mixture of 15% di-, 45% tri-, and 28% tetradeuterio-**2** (determined by mass spectral analysis; see Experimental Section) with 5,6,7-trideuterio-**2** (the three deuteriums are in a cis relationship) being the major product. The ^{1}H NMR spectrum comprises five singlets due to the 6-methyl group, H-5ax, H-5eq, H-7ax, and H-7eq in the ratio 3.0:0.79:0.06:0.51:0.1. The integration of the signals due to H-7 and H-5 shows that ca. 30% of the product has incorporated two deuteriums at C-7 which is in agreement with the mass spectral analysis. A control experiment which involved incubating **2** in trifluoroacetic acid-*d* showed the incorporation of ca. 7% deuterium after 24 h. In addition there is ample literature precedence for H–D exchange in aliphatic hydrocarbons in the presence of platinum dioxide catalyst in a deuterium atmosphere.[13] The integration of H-5ax relative to H-5eq indicates that the reduction proceeds with a ca. 93% stereospecificity with all deuteriums being introduced from the side of the molecule opposite the methyl group. This result contrasts with a lack of stereoselectivity seen in the reduction of the corresponding pterin derivative[14] but is consistent with previous observations of stereoselectivity in the hydrogenation of pyridine derivatives.[12]

Electrochemical studies show 6-methyl-5-deazatetrahydropterin (**2**) to have a much higher oxidation potential than tetrahydropterin derivatives.[15] The oxidation peak potential for **2** under several conditions is shown in Table II. The electrochemical oxidation of **2** is an irreversible two-electron process. In accord with these data **2** is found to be inert toward molecular oxygen but is oxidized by, for example, ceric ammonium nitrate and lead dioxide.[16]

6-Methyl-5-deazatetrahydropterin reacts with a variety of electrophilic reagents to give in each case the appropriate 4a adduct. These reactions are summarized in Scheme I and will be discussed in greater detail in the subsequent text. The reactions of **2** with *N*-halosuccinimide, bromine, and peroxy acid probably proceed either by an electrophilic aromatic substitution process or by addition across the 4a,8a-double bond with a subsequent elimination reaction affording the observed product (Scheme II). It is possible to exclude a mechanism involving the initial oxidation of **2** to a quinone methide (**7**) (analogous to the quinonoid intermediate believed to be involved in tetrahydropterin oxidation)[2-4] since with deuterated **2** no loss of the deuterium label from C-5 is observed in the product (the NMR and mass spectral analysis are given in the Experimental Section).

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Table I. The 360-MHz ^{1}H NMR Data[a]

compd	chemical shift (±0.01 ppm)						coupling constant (±0.5 Hz)						
	CH_3	H-5ax	H-5eq	H-6	H-7ax	H-7eq	J_{5ae}	J_{5a6}	J_{5e6}	J_{7ae}	J_{7a6}	J_{7e6}	J_{CH_36}
2[b,c]	0.95	1.90	2.45	1.91	2.89	3.31	−14	11	3	−12.5	9.2	3.7	6.3
trans-**3**[c]	1.07	1.91	2.49	2.65	3.17	3.73	−15.1	12.5	2.2	−14.7	11.4	5.8	6.6
trans-**4**[c]	1.08	2.00	2.48	2.58	3.09	3.69	−14.7	12.5	2.2	−14.7	11.4	5.5	6.6
trans-**5**[c]	1.04	1.65	2.27	2.40	3.04	3.62	−14.3	13.2	2.2	−14.7	11.0	5.9	6.6
cis-**4**[d]	1.10	2.27	2.80		3.37	3.57	−15.1	7.8	8.4	−14.3	10.6	5.5	7.0
cis-**5**[d]	1.08	1.96	2.94		3.32	3.49	−15.1	6.8	8.8	−13.7	10.3	5.4	6.8

[a] The spectra were recorded in D_2O. Chemical shifts are reported in ppm from Me_4Si. [b] The chemical shift of H-6 and the coupling constants J_{5ae}, J_{5a6}, and J_{5e6} are estimates based on a computer simulation (reference 10). The error in these values is ~±1 Hz. [c] W coupling between H-5ax and H-7ax is apparent in the spectra of **2** and *trans*-**3**, **-4**, **-5**. Its magnitude is ~1.5 Hz in **2** and ~0.5 Hz in *trans*-**3**, **-4**, **-5**. No similar coupling is observed in the spectra of *cis*-**4** and **-5**. [d] The H-6 hydrogen is obscured by the resonances due to the trans isomer.

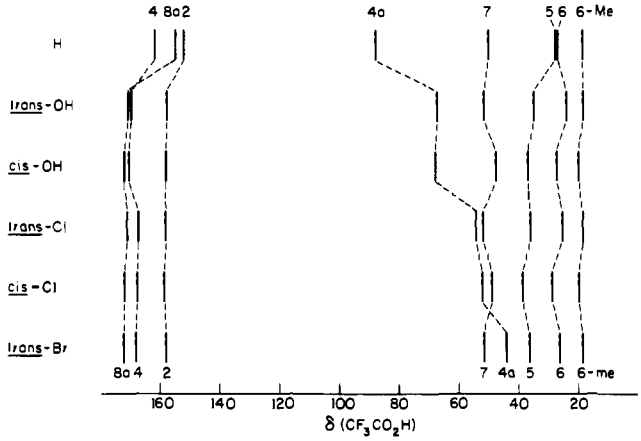


Figure 1. The ^{13}C NMR spectra of (from top to bottom) **2**, *trans*- and *cis*-**5**, *trans*- and *cis*-**4**, and *trans*-**3**. Chemical shifts are reported in ppm from Me_4Si.

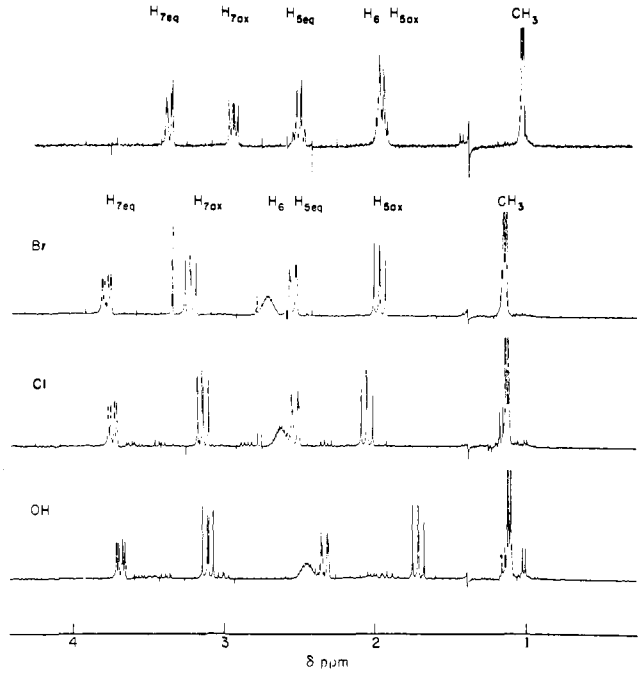


Figure 2. The 360-MHz ^{1}H NMR spectra of (from top to bottom) **2** and *trans*-**3**, **-4**, and **-5**. The spectra of the chloro and hydroxy adducts **4** and **5** also show contamination with the corresponding cis isomer. The spectrum of **5** also shows a trace amount of a decomposition product.

4a-Adducts: Spectra, Structure, and Stereochemistry. Proof of the structure and stereochemistry of the products of the above mentioned reactions rests primarily on NMR data. The ^{13}C NMR spectra of the cis and trans isomers (the relationship between the substituents at C-4a and the 6-methyl) of **3, 4,** and **5** are shown schematically in Figure 1. Assignment of the resonances for the sp^3 carbons follows from an examination of the chemical shifts (Figure 1) and the multiplicities observed in the gated decoupled spectrum of **3** (Figure 3). The methyl group, C-5, and C-6 are readily distinguished since they appear as a quartet, triplet, and doublet, respectively. The C-7 is a triplet and displays a chemical shift typical of a carbon α to nitrogen.[17] The chemical shift of C-4a in **3, 4,** and **5** is characteristic of a sp^3 carbon bearing a bromo, chloro, and hydroxyl substituent, respectively; the observed chemical shift may be calculated within ±2 ppm by adding the literature substituent parameters for bromo, chloro, and hydroxyl[17c,18] to a base value of ~20 ppm. The sp^2 carbons can be distinguished since the peak due to C-8a shows line broadening (ca. 8 Hz, see Figure 3) due to long-range coupling to the C-7 hydrogens. Notably no line broadening is observed in the peak due to C-4 since this carbon is isolated from the C-5 hydrogens by the 4a substituent (line broadening is observed for C-4 in the gated decoupled spectrum of **2**). Sites C-2 and C-4 are distinguished by their chemical shift. The relative insensitivity of the chemical shift of all carbons except C-4a to the nature of the 4a substituent (see Figure 1) is indicative of the close structural and stereochemical similarity of **3, 4,** and **5**.

The 360-MHz ^{1}H NMR spectra of **3, 4,** and **5** show first-order splitting patterns and both chemical shifts and coupling constants (shown in Table I) can be evaluated directly from the spectra (Figure 2). A study of the molecular models of the cis and trans isomers of **3, 4,** and **5** shows that the trans isomer may readily adopt a pseudo-chair conformation with the 4a substituent axial and the 6-methyl group equatorial. On the other hand, the cis isomer should favor a non-chair conformation order to avoid—if the molecule adopts a chair conformation—a 1,3-diaxial interaction between the 4a substituent and the 6-methyl group or the strain introduced into the pyrimidine ring with the 4a group equatorial. This strain can be largely relieved by the molecule assuming a pseudo-boat conformation. The coupling constants and chemical shifts for the major isomer formed during the synthesis are consistent with it adopting a chair conformation;[11] thus, this isomer is assigned the trans configuration. The observation of "J_{5a6}" ≲ "J_{5e6}" (Table I) demonstrates that the minor isomer has a non-chair conformation; thus, it is assigned the cis configuration. The coupling constants observed are consistent with the distorted boat conformation predicted by model studies.

Direct evidence for the stereochemistry of the major isomer of **5** comes from the X-ray structure of its decomposition product (vide infra) which shows that the hydroxyl substituent and the 6-methyl group have a trans relationship to each other. This assignment of stereochemistry is also consistent with the expected influence of steric factors in the synthesis (i.e., approach of the reagent preferentially from the less hindered side opposite the methyl group). It is notable in this respect that there is a direct relationship between the size of the attacking

Table II. Half-Peak Potentials[a]

compd	medium	pH	$E_{p/2}$ (V vs. SCE)[b,c] oxidation (A)	reduction (C)	electrode
$DMPH_4$	borate	9.0	−0.23	−0.19	HMDE[d]
	borate	9.0	−0.14	−0.30	CPE
2[e,f]	borate	9.0	+0.61		CPE
	phosphate	3.0	+0.82		CPE
	phosphate	7.0	+0.69		CPE
	phosphate	12.0	+0.42		CPE
	0.1 M TEAF/CH_3CN		+0.81		GCE[g]
5	phosphate	7.0		−1.04	HMDE
5[h]	phosphate	7.0		−1.49	HMDE

[a] The following abbreviations are used: $DMPH_4$, 6,7-dimethyltetrahydropterin; *q*-$DMPH_2$, *quinonoid*-6,7-dimethyldihydropterin; HMDE, hanging mercury drop electrode; CPE, carbon paste electrode; GCE, glassy carbon electrode; TEAF, tetraethylammonium tetrafluoroborate; SCE, standard calomel electrode. [b] Aqueous cyclic voltammetric measurements were carried out in 0.1 M buffers by using a scan rate of 154 mV/s. [c] Experiments in acetonitrile were carried out by using a scan rate of 200 mV/s and an acetonitrile $Ag/AgNO_3$ reference electrode (+0.337 V vs. SCE). [d] Kwee and Lund (reference 15) report $E_{p/2,A} = -0.26$ V and $E_{p/2,C} = -0.22$ V vs. SCE for the $DMPH_4/q$-$DMPH_2$ couple under these conditions. [e] The free base form of **2** was used. [f] The oxidation of **2** occurs at potentials too positive to be examined by using HMDE. [g] Coulometry using GCE in acetonitrile shows the oxidation of **2** to be a two-electron process. [h] This solution contains the immediate decomposition product of **5** (λ_{max} 232).

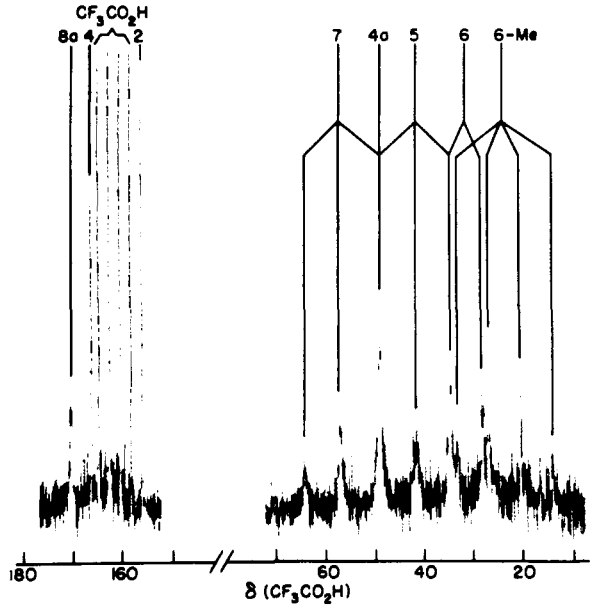
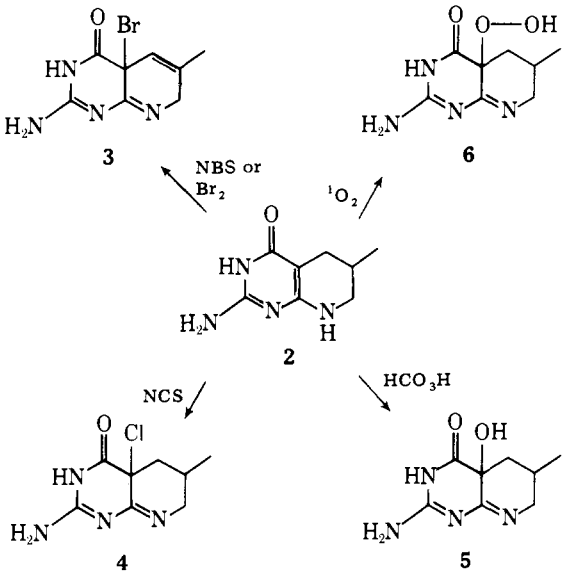
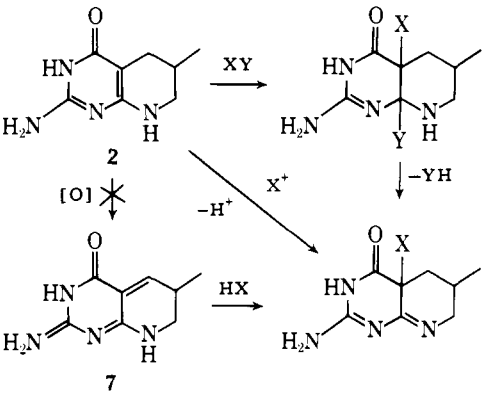


Figure 3. Gated-decoupled ^{13}C NMR spectrum of **3**.

Scheme I



Scheme II



reagent and the degree of stereospecificity observed in the syntheses of **3**, **4**, and **5**.

The UV spectra of **3**, **4**, and **5** while similar in form (Figure 4) show features which are diagnostic of the nature of the 4a substituent. The position of the absorption maxima shifts to longer wavelength and the extinction coefficient decreases within the series where the 4a substituent is an alkyl,[19a] hydroxyl, peroxy, chloro, or bromo group. The spectra are pH dependent allowing the pK_a's of **2**, **3**, **4**, and **5** to be determined (3.6 ± 0.1, 5.2 ± 0.1, 5.3 ± 0.1, and 6.1 ± 0.2,[19b] respectively).[19c] The greater basicity of **3**, **4**, and **5** in relation to **2** may reflect the disruption of the aromatic pyrimidine ring and a decreased delocalization of the nitrogen lone pair responsible for the pK_a.

Bromo and Chloro Adducts. 6-Methyl-5-deazatetrahydropterin (**2**) reacts rapidly and quantitatively with *N*-bromo- or *N*-chlorosuccinimide, or bromine to form the corresponding 4a-halo adduct (**3** and **4**). The reaction occurs with apparent equal efficiency using either methylene chloride, acetonitrile, methanol, acetic acid, or water (with bromine) as solvent and requires 1 equiv of the halogenating agent. Samples of the halo adducts (**3**, **4**) required for characterization and further study were prepared by reacting **2** with the appropriate *N*-halosuccinimide in methanol containing trifluoroacetic acid. The acid is required both to enhance the solubility of **2** in methanol and to ensure the product is formed in the protonated form (the free

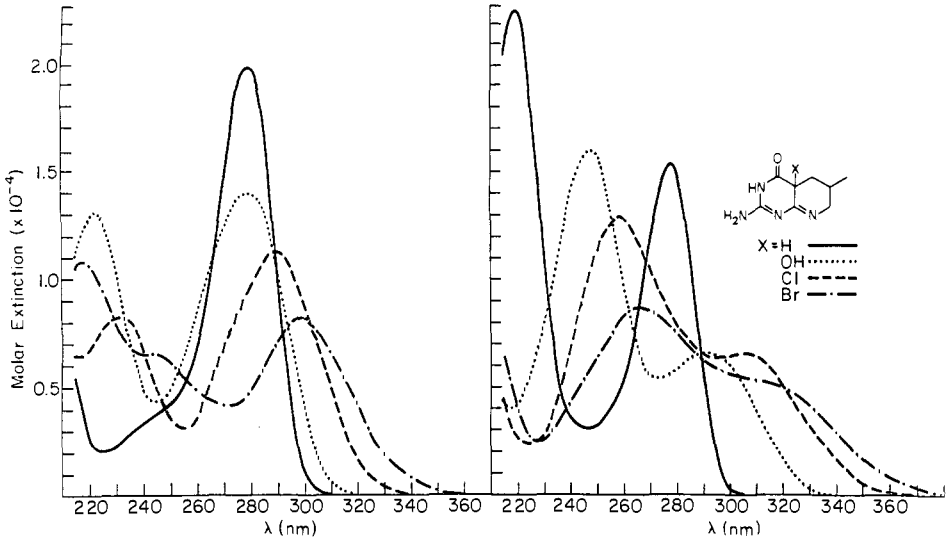


Figure 4. UV spectra of **2** (—), *trans*-**3** (- - -), *trans*-**4** (---), and *trans*-**5** (···) in (left) 0.1 N HCl and (right) 0.01 M Tris, pH 8.06.

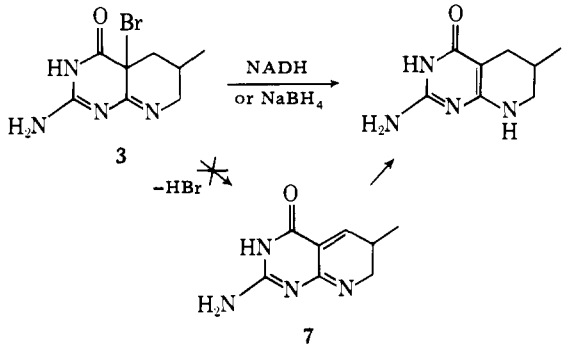
base form of **3** is very labile and is susceptible to decomposition during workup). The bromo adduct (**3**) is shown by LC (high pressure liquid chromatography) and NMR to be a single isomer (>98% trans), whereas the chloroadduct (**4**) is formed as a mixture of trans and cis isomers in the ratio 9:1.

The bromo adduct (**3**) is unstable in solution and reacts with added nucleophiles or solvent in an apparent substitution reaction. In alkaline aqueous solution or in methanolic potassium hydroxide, **3** is observed by LC and UV to be converted into the hydroxy adduct (**5**), and in dilute hydrochloric acid into the chloro adduct (**4**). The bromo adduct (**3**) also reacts with hydride reducing agents (NADH, $NaBH_4$) to form **2**. The kinetics of the reduction have been examined and are found to be second order with $k \sim 3.9 \times 10^{-2}$ M^{-1} s^{-1} for NADH (pH 8.1, 25 °C). An elimination–addition mechanism (Scheme III) for the reaction is excluded by the observation that no deuterium incorporation is observed by mass spectrometry when the reaction is carried out by using sodium borodeuteride and/or with deuterium oxide as solvent. If a quinone methide (**7**) is an intermediate in the reduction, deuterium must be incorporated at C-5 under one of the above conditions.

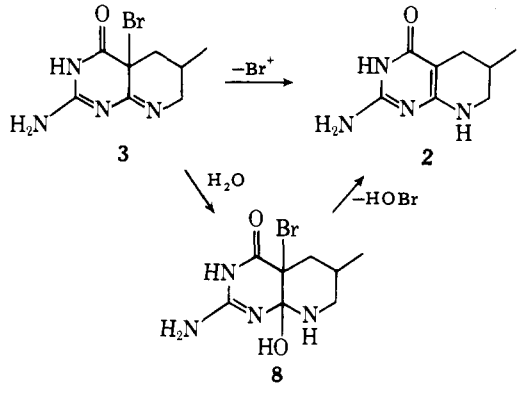
The bromo adduct (**3**) is also observed to undergo a slow conversion into **2** in the absence of any added nucleophiles. Plausible mechanisms for the process (Scheme IV) include (a) formation of the hydrate **8** and subsequent elimination of hydrobromous acid (a similar mechanism is proposed to account for the ready dehalogenation of 5,5-dihalogenopyrimidines[20]) and (b) the direct transfer of Br^+ to some acceptor molecule. These mechanisms will also account for the observation that **3** is able to brominate 1,3-dimethoxybenzene (Scheme V). The bromination reaction requires an acidic medium; optimum conditions for the reaction use 10^{-2} M hydrobromic acid as solvent at room temperature to give a 70% yield of 1-bromo-2,4-dimethoxybenzene (based on **3**; determined by GCMS and LC). The reaction is also observed (~15% yield) by using dilute hydrochloric acid as solvent but is complicated by a side reaction of **3** to form the chloro adduct **4**. The chloro adduct (**4**) does not perform a chlorination under these conditions.

Hydroxy Adduct. The reaction of performic acid (formed in situ from the reaction of hydrogen peroxide and the formic acid solvent)[21] is shown by LC and UV (in acetonitrile) to be quantitative affording the hydroxy adduct (**5**) as a mixture of the cis and trans isomers in the ratio 2:3. When carried out on a preparative scale, the yield of product is limited by some instability of **5** to the conditions used in working up the reaction mixture (see Experimental Section). The major (trans) isomer is isolated in >95% purity (by NMR and LC) and 30% yield by repeated crystallization from methanol–ether leaving the cis isomer to be recovered from the recrystallization residue in ~60% purity (the major contaminant is the trans isomer).

Scheme III



Scheme IV



Scheme V

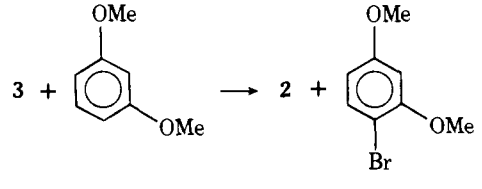


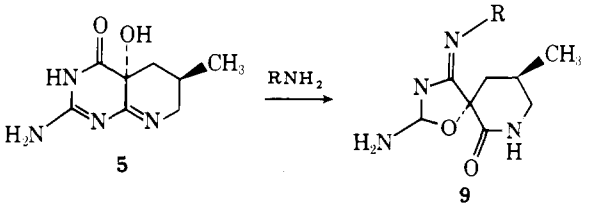
Table III. Second-Order Rate Constants (25 °C) for the Conversion of Thioxane to Its Sulfoxide in Methanol

compd	rate constant
CH_3CO_2OH	3.75×10^{-6} [a]
H_2O_2	9.38×10^{-5} [a]
6	5.2×10^{-2} [b]
4aFlCD$_3$OOH[c]	6.6×10^{-1} [a]

[a] Values abstracted from reference 28. [b] Using 10-50 mM thioxane and 10 μM **6**. [c] The abbreviation 4aFlCD$_3$OOH stands for 4a-hydroperoxy-5-trideuteriomethyl-3-methyllumiflavin.

In aqueous solution within the pH range 2-10, **5** is observed to undergo a facile decomposition. The process shows first-order kinetics and is pH independent at pH <4 and >6.5 at 25 °C ($k_{obsd} = 7.0 \times 10^{-5}$ and 1.6×10^{-3} s^{-1}, respectively). The immediate product of the reaction, which shows a single peak on LC and has λ_{max} 235 nm (ε 15 000), is itself extremely labile and is not readily isolated or characterized.

In the presence of methoxyamine or semicarbazide at pH 4.8, **5** is transformed into the corresponding spiro compound (**9**). The semicarbazone derivative (**9b**) is crystalline and its



5 → **9**

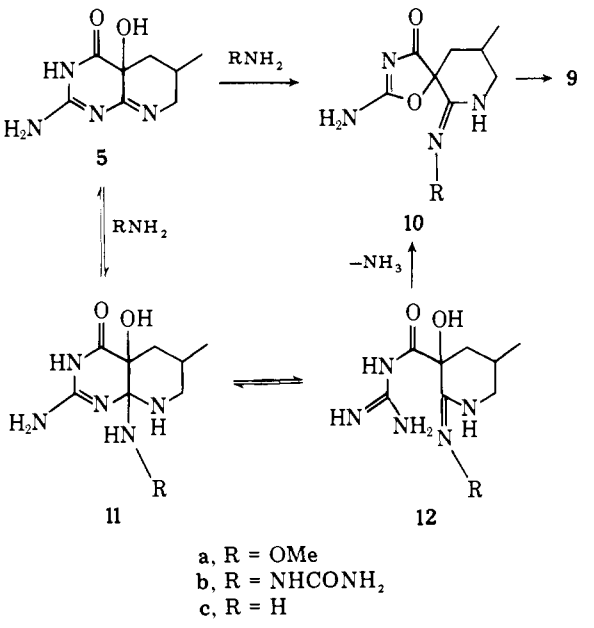
a, R = OMe
b, R = NHCONH$_2$

structure and stereochemistry have been proved by X-ray analysis (see Experimental Section). Attempts to form **9** by treating a solution of the hydroxy-adduct decomposition product with semicarbazide or methoxyamine were unsuccessful. This result shows that the "trapping reagent" does not simply react with an oxazolidinedione species to form **9** but rather must have a direct role in its formation from **5** (vide infra). The reaction **5** → **9** has also been carried out by using $H_2{}^{18}O$ in the solvent. Mass spectral analysis of the product shows no ^{18}O incorporation. This result shows that the 4a hydroxyl of **5** becomes O-1 in the oxazolone ring of **9** and also indicates that the piperidone oxygen is derived during the workup procedure.

Two mechanisms (Scheme VI) will account for the above observations: (a) attack by the 4a hydroxyl of **5** at C-2 and subsequent or concerted cleavage of the N-1, C-2 bond to give the amidine **10c**; and (b) formation of the orthoamide **11**, followed by the cleavage of the C-8a, N-1 bond and cyclization by attack of the 4a hydroxyl at C-2 to give **10a** or **b**. The piperidone oxygen of **9** is derived by hydrolysis of the amidino group of **10** during the workup procedure. Mechanism a has precedence in the known rearrangement of dialluric acids (5-hydroxybarbituric acids) to give oxazolidine-2,4-diones in alkaline media.[22,23] Mechanism b has analogy in the rearrangement of lumazine and flavin derivatives which react with initial cleavage of the "C-8a, N-1" bond but cyclize to give a spirohydantoin.[5,6,23,24] Since C-4a is not involved directly in the rearrangement of **5**, the stereochemistry of **9** serves to prove the configuration proposed for the major isomer of **5** (i.e., the 4a hydroxyl substituent is trans to the 6-methyl group).

The hydroxy adduct (**5**) is inert toward reduction using conditions under which the halo adducts (**3** and **4**) are rapidly converted into **2** (for example, using $NaBH_4$ and NADH). The compound **5** is, however, reduced electrochemically (see Table II) to **2** (identified by its oxidation peak in the cyclic voltammogram). The immediate decomposition product of **5** is also reduced electrochemically to **2**. The oxidation of **2** is observed by cyclic voltammetry only after partial reduction of **5** (or its decomposition product), thus eliminating the possibility that the **2** observed is an impurity. While the electrochemical data are not sufficient criteria for assigning a definite structure to the immediate decomposition product of **5**, any structure proposed for this compound must be capable of being reduced to **2**. Thus, this compound cannot be an oxazolidinedione derivative (analogous to **9**) since the latter's formation involves the loss of an amino group, but might be the 8a hydrate or an acyclic species in equilibrium with it.

Scheme VI



a, R = OMe
b, R = NHCONH$_2$
c, R = H

Peroxy Adduct. 6-Methyl-5-deazatetrahydropterin (**2**) reacts with singlet oxygen (generated photochemically by using immobilized Rose Bengal as a sensitizer)[25] in methanol to form a highly reactive hydroperoxide (**6**). The nature of this product (**6**) is indicated by the UV spectrum, which is identical with that of the hydroxy adduct (**5**) with the exception that the absorption maxima are shifted to longer wavelengths by ca. 2 nm, and by the observation that it is reduced to form **5** (a mixture of trans and cis isomers in the ratio 3:2) under a variety of conditions. The purity of the peroxide, gauged by the spectrophotometric determination of the I_3^- formed by reaction with potassium iodide in methanol,[26,27] is greater than 80%. Attempts to determine the peroxide electrochemically were unsuccessful, only the reduction of **5** was observed.

The high reactivity of **6** is seen in the rate of its reaction with thioxane and with iodide in methanol. The kinetics of the former reaction have been examined with pseudo-first-order conditions and the rate constant is compared in Table III with values for other peroxides.[28] The only identifiable products formed from **6** in these reactions are the hydroxy adduct (**5**) (in >80% yield) and its rearrangement product (identified by LC and UV). The peroxide **6** is also rapidly converted into **5** on dissolution in an aqueous medium.

Biological Activity. 6-Methyl-5-deazatetrahydropterin has been shown to be a competitive inhibitor of both rat liver phenylalanine hydroxylase and brain tyrosine hydroxylase.[1] No cofactor activity was found; neither oxygen uptake nor tyrosine formation was observed.

Tests[29] conducted with *trans*-**3**, **-4**, and **-5** and phenylalanine hydroxylase show that these compounds neither inhibit nor inactivate the enzyme (Table IV)[30-33] despite the fact that **5** may be a product analogue[2-4] and **3** and **4** are active halo-

Table IV. Assays with Phenylalanine Hydroxylase[a,b]

compd	initial rate[c]	tyrosine formed[d]
	1.0 (1.2)[e]	1.0
2	0.6	0.85
3	0 (0.6)[f]	0.08 (0.72)[f]
4	1.0[e]	1.17[e]
5	1.0[e]	1.22[e]

[a] The compound (**2–5**) was incubated with all assay components for 10 min prior to the addition of 6,7-dimethyltetrahydropterin. [b] The compound (**2–5**) and 6,7-dimethyltetrahydropterin were used in equimolar amounts (0.03 mM). [c] Initial rate of enzyme reaction in the presence of **2–5** relative to that in the absence of **2–5** (see Experimental Section). [d] Amount of tyrosine formed in the presence of **2–5** relative to that in the absence of **2–5** (references 32, 33). [e] Catalase was present in the assay. [f] After the addition of a second equivalent of 6,7-dimethyltetrahydropterin.

genating agents and potential irreversible inhibitors. The assays are, however, complicated by the instability of the adducts. Under the assay conditions, the hydroxy adduct (**5**) rearranges with $t_{1/2} \sim 8$ min, whereas the bromo adduct (**3**) reacts rapidly and quantitatively with 6,7-dimethyltetrahydropterin to give the oxidized cofactor and **2** (any inhibition caused by the latter compound can be rationalized in terms of the amount of **2** formed). The experiments also show that phenylalanine hydroxylase has no significant influence on the rate of decomposition of the adducts.

The peroxy adduct (**6**) is a model of the active hydroxylating species (or its immediate precursor) proposed by several workers. Accordingly this compound (20 μM) was incubated with phenylalanine hydroxylase under standard assay conditions[30] (both with and without NADH and *quinonoid*-dihydropterin reductase) to test for tyrosine formation. The fact that no cofactor activity was observed may be caused by a diminished binding to the hydroxylase so that the turnover of **6** by the enzyme is slow relative to its decomposition under the assay conditions ($t_{1/2} \approx 5$ min).

Conclusions

The observation of 4a-adduct formation in the reactions of various electrophilic reagents with the 6-methyl-5-deazatetrahydropterin lends support to the hypothesis that the natural cofactor might participate in the biological hydroxylation through a 4a-peroxy adduct. The ability of the bromo derivative (**3**) to brominate dimethoxybenzene and the increased reactivity of the peroxy species (**6**) relative to hydrogen peroxide in the oxidation of thioxane also is supportive. Of considerable interest are the decomposition products of the hydroxy adduct. Both the electrochemistry and X-ray results are in accord with transient and putative 4a,8a-addition complexes suggesting that the 8a carbon has increased sensitivity to nucleophilic attack after two-electron oxidation of the tetrahydropterin and might be important in a reductive reaction. Although 6-methyl-5-deazapterin functions as a competitive inhibitor of two hydroxylase enzymes, the high solution reactivity of its 4a derivatives coupled with weak binding apparently precludes their effectiveness as inhibitors.

Experimental Section

The 100-MHz ^{1}H NMR spectra were obtained with a Jeol PS-100-FT spectrometer and 360-MHz ^{1}H NMR spectra with a Bruker WH360 spectrometer; ^{13}C NMR were recorded by using a Varian CFT-20 or a Jeol PS-100-FT instrument. All spectra are referenced to Me_4Si (0 ppm) as standard. Ultraviolet spectra were obtained with a Cary 118 or 14 spectrophotometer. Fluorescence spectra were recorded with a Perkin-Elmer MPF44A instrument. MS were recorded with a AEI MS-9 mass spectrometer. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. pH measurements were made with a Radiometer 22 instrument equipped with a scale expander. Elemental analyses were performed by M-H-W Laboratories, Phoenix, Ariz.

Table V. LC Retention Times (in minutes)

compd	column ODS[a]	column SCX[b]
solvent[c]	0.6	0.6
1	5.6	
2	8.6	27.5
trans-**3**	13.4	
trans-**4**	14.4	
cis-**5**	6.2 (11.6)[d]	6.3
trans-**5**	6.4 (12.4)[d]	7.5
5[e]	6.9 (14.8)[d]	
9a	1.1	3.5
9b	1.2	3.9

[a] Whatman ODS-2 reverse phase column 4.6 mm × 25 cm, eluant MeOH/H_2O/NaDodSO$_4$/H_2SO_4 (50:50:0.02:0.04, v/v/w/v); 2 mL/min. [b] Whatman SCX cation exchange column 4.6 mm × 25 cm, eluant 0.05 M ammonium formate, pH 3.3; 2 mL/min. [c] Time from injection of sample to solvent front. [d] As in footnote *a* above but using MeOH/H_2O/NaDodSO$_4$/H_2SO_4 (43:57:0.02:0.04, v/v/w/v) as eluant. [e] Immediate decomposition product of **5**.

Methanol was distilled from magnesium methoxide, acetonitrile from calcium hydride, and ether from lithium aluminum hydride. Doubly distilled deionized water was used unless stated otherwise.

Gas Chromatography (GC) and GCMS. Analytical GC was carried out with a Finnigan 9500 instrument and the following columns: (a) 5 ft × 2 mm, 3% SP2100 on Supelcoport (100–120 mesh); (b) 5 ft × 2 mm, 3% OV17 on Gas-Chrom Q (100–200 mesh). The latter column was treated with Rejuv-8 (Supelco) prior to the analysis of silyl derivatives. Helium (20 mL/min) was used as the carrier gas. GCMS was carried out on a Finnigan 3200 instrument equipped with a Finnigan 6000 data system with the same columns and conditions used for analytical GC.

The compounds **1–5** were converted to their silyl derivatives for GC analysis. Approximately 50 μg of the sample was suspended in 0.2 mL of acetonitrile in a septum-sealed vial under nitrogen and 50 μL of *N,N*-diethyltrimethylsilylamine (Aldrich) was added by syringe. The mixture was heated at 60 °C for 30 min and cooled, and the acetonitrile phase was analyzed by using column a (200 °C for **1** and **2**; 280 °C for **5**). The following retention times were observed: **1** (disilylated; GCMS *m/e* 320 (M^+), 4.8 min; **2** (monosilylated; GCMS *m/e* 252 (M^+)), 6.4 min; **2** (disilylated; GCMS *m/e* 324 (M^+)), 5.4 min; **2** (disilylated; GCMS *m/e* 324 (M^+)), 5.1 min; **2** (trisilylated; GCMS *m/e* 396 (M^+)), 4.0 min; **5** (disilylated, GCMS *m/e* 340 (M^+)), 7.9 min; **5** (disilylated, GCMS *m/e* 340 (M^+)), 8.4 min. Compounds **3** and **4** were reduced to **2** under the conditions used for silylation. The cis and trans isomers of **5** were not resolved. The relative yield of the various silylated derivatives of **2** was dependent on the reaction conditions and the time the sample was allowed to stand before analysis.

High Pressure Liquid Chromatography (LC). Separations were carried out by using an Altex 100 solvent metering system, an Altex 400 solvent programmer, and an Altex 153 UV detector operating at 254 nm. All routine analyses were carried out by using ion pair chromatography[34] and sodium dodecyl sulfate (NaDodSO$_4$) as the ion pair reagent. The retention times observed and the conditions used are summarized in Table V. The cation-exchange column was used to confirm product identity in the case of difficult separations.

Electrochemistry. The cyclic voltammetric measurements were made with a Bioanalytical Systems CV-1A potentiostat (Bioanalytical Systems, West Lafayette, Ind.) by using a scan rate of 154 mV/s. The current-potential curves were recorded on a Hewlett-Packard 7035B X-Y recorder. Coulometry was performed with a Princeton Applied Research 170 multipurpose instrument by using a Pine Analytical Rotator ASR at 400 rpm.

A Metrohm AG CH-9100 hanging mercury drop electrode (HMDE) and a Bioanalytical Systems carbon paste electrode (CPE) were used for cyclic voltammetric studies. The reference electrode was a saturated calomel electrode (+0.25 V vs. NHE) to which all potentials cited are referred. The counter electrode was a platinum wire. Buffers were prepared from reagent grade salts by using triply distilled

deionized water. A glassy carbon (Tokai Electrode Manufacturing Co., Tokyo) electrode (GCE) was used for electrochemistry in acetonitrile. The acetonitrile (Burdick and Jackson) was distilled under nitrogen from calcium hydride. The electrolyte was 0.1 M tetraethylammonium tetrafluoroborate (Southwestern Analytical Chemicals, Austin, Texas). Measurements were made with a $Ag/AgNO_3$ acetonitrile reference electrode (+0.337 V vs. SCE). The results of these experiments are reported in Table II.

6-Methyl-5-deazatetrahydropterin (2). A solution of 2.0 g of 6-methyl-5-deazapterin (**1**)[7] in 15 mL of trifluoroacetic acid was hydrogenated in the presence of 100 mg of platinum dioxide under 3 atm of hydrogen. After 3 h, the catalyst was removed by filtration, the solvent evaporated, and the residue dried over potassium hydroxide to afford 4.6 g (99%) of **2** as the ditrifluoroacetate salt. The product showed one peak on LC. Recrystallization of this material from either methanol or ethanol-water-acetone (1:1:3) gave the analytically pure monotrifluoroacetate salt; mp >360 °C; ^{13}C NMR (coupled, CF_3CO_2H) δ 18.0 (q, J = 126 Hz, CH_3), 26.5 (d, J = 134 Hz, C-6), 26.9 (t, J = 135 Hz, C-5), 49.3 (t, J = 143 Hz, C-7), 87.6 (s, C-4a), 151.7 (s, C-2), 154.3 (br s, C-8a), 161.7 (br s, C-4). Anal. Calcd for $C_{10}H_{11}F_3N_4O_3$: C, 40.8; H, 4.5; N, 19.0. Found: C, 40.7; H, 4.2; N, 19.1.

A suspension of 500 mg of the above monotrifluoroacetate salt in 5 mL of saturated potassium carbonate solution was stirred at 0 °C until carbon dioxide evolution appeared complete. The mixture was then filtered and the product dried over phosphorus pentoxide and recrystallized from methanol to give 240 mg (78%) of the free base of **2** as white flakes: mp 284–286 °C; MS m/e 180 (M^+). The ^{13}C NMR of **2** is shown in Figure 1, the 360-MHz ^1H NMR in Table I and Figure 2, and the UV at pH 1 and 8 in Figure 4.

Deuterated 6-Methyl-5-deazatetrahydropterin (2). A solution of 150 mg of **1** in 3 mL of trifluoroacetic acid-d was shaken in a Parr hydrogenation apparatus under 3 atm of deuterium in the presence of 20 mg of platinum dioxide. After 16 h, the catalyst was removed by filtration and the solvent evaporated to afford 350 mg (96%) of the ditrifluoroacetate salt. This material showed an identical UV spectrum (Figure 4) and LC trace with the those of nondeuterated material described above: 100-MHz ^1H NMR (CF_3CO_2H) δ 1.2 (3 H, s, CH_3), 2.1 (0.79 H, s, H-5ax), 2.9 (0.06 H, s, H-5eq), 3.1 (0.51 H, s, H-7ax), 3.6 (0.1 H, s, H-7eq).

Deuterium Analysis. (a) MS. Conventional 70-eV mass spectrometry cannot be used to determine the extent of deuterium labeling of **2** due to an intense M − 1 fragment ion in the spectrum (see below). Accordingly the mass spectrum was recorded at 12 eV. MS (70 eV, of unlabeled **2**) m/e 181, 10%; 180 (M^+), 100%; 179, 43%; MS (12 eV, of unlabeled **2**) m/e 181, 12%; 180 (M^+), 100%; 179, 3.5%; MS (12 eV, of labeled **2**) m/e 186, 4%; 185, 14%; 184, 63%; 183, 100%; 182, 25%; 181, 4%; 179–180, <1%. The peak intensities have an error of <±2%. Deconvolution of these data in the usual manner shows the labeled **2** to comprise 29% tetra-, 56% tri-, and 15% dideuterio-**2**.

(b) GCMS. Although the mass spectrum of the disilyl derivative of **2** shows an intense M − 1 fragment ion, it is still possible to obtain a reliable determination of the deuterium content of **2** by examining the M − 15 fragment peak. GCMS (unlabeled **2**) m/e 313, 1.1%; 312, 3.0%; 311, 10.6%; 310, 27.9%; 309 (M − 15), 100.0%; 308, <1%; GCMS (labeled **2**) m/e 316, 1.9%; 315, 8.0%; 314, 25.9%; 313, 74.2%; 312, 100.0%; 311, 25.2%; 310, 4.0%; 309, 2.9%; 308, <1%. The error in the peak intensities is <±0.5%. These data show labeled **2** to comprise 28% tetra-, 57% tri-, and 15% dideuterio-**2**.

4a-Bromo-6-methyl-5-deazatetrahydropterin (3).[35] (a) A solution of 400 mg of **2** and 400 μL of 47% HBr in 1 L of water was treated with 355 mg of bromine in a foil-covered flask. The resulting colorless solution was lyophilized to afford 740 mg (94%) of a pale yellow powder. In the absence of added HBr, significant decomposition (to form **2**) occurs in the workup procedure. The product is identical in all respects apart from the salt form with the material described in the procedure (b) below.

(b) N-Bromosuccinimide (400 mg) was added in one portion to a solution of 400 mg of **2** in 500 mL of methanol containing 0.5 mL of trifluoroacetic acid in a foil-covered flask. After 1 h, the solvent was evaporated to leave a colorless oil which was triturated with ether to afford a white powder. This material was washed four times with 2 mL of acetonitrile (to remove succinimide) to afford analytically pure **3** (800 mg, 97%) as the trifluoroacetate salt: mp >360 °C; ^{13}C NMR (coupled, CF_3CO_2H) δ 18.0 (q, J = 127 Hz, CH_3), 25.8 (d, J = 131 Hz, C-6), 36.0 (t, J = 147 Hz, C-5), 43.4 (s, C-4a), 51.2 (t, J = 147 Hz, C-7), 157.7 (s, C-2), 167.7 (s, C-4), 171.8 (br s, C-8a)—all coupling constants ±5 Hz. Anal. Calcd for $C_{10}H_{10}BrF_3N_4O_3 \cdot 0.5H_2O$: C, 31.6; H, 3.2; Br, 21.0; N, 14.7. Found: C, 31.5; H, 2.9; Br, 21.0; N, 14.7.

4a-Chloro-6-methyl-5-deazatetrahydropterin (4).[35] By using the procedure (b) described above, 500 mg of **2** was treated with 370 mg of N-chlorosuccinimide in methanol-trifluoroacetic acid to afford 890 mg (98%) of **4** as the trifluoroacetate salt: mp >360 °C. The product was shown by ^{13}C and ^1H NMR to contain the cis and trans isomers in the ratio 1:9. The trans isomer has: ^{13}C NMR (CF_3CO_2H) δ 17.9 (CH_3), 24.6 (C-6), 35.7 (C-5), 51.3 (C-7), 53.9 (C-4a), 158.0 (C-2), 166.9 (C-4), 171.0 (C-8a)—peak assignments based on analogy with those of **3** and **5**. Anal. Calcd for $C_{10}H_{10}ClF_3N_4O_3$: C, 36.5; H, 3.7; Cl, 10.8; N, 17.1. Found: C, 36.8; H, 3.6; Cl, 10.8; N, 16.8. The cis isomer has: ^{13}C NMR (CF_3CO_2H), δ 17.6 (CH_3), 28.2 (C-6), 38.2 (C-5), 48.5 (C-7), 51.8 (C-4a), 158.4 (C-2), 167.5 (C-4), 171.8 (C-8a)—peak assignments based on analogy with those for **3** and **5**.

Reactions Using Deuterated 2. The reactions to form **3** and **4** were also conducted by using deuterated **2** as substrate and the identical reaction conditions: 100-MHz ^1H NMR (labeled **3**, CF_3CO_2H) δ 1.1 (3 H, s, CH_3), 1.9 (0.74 H, s, H-5ax), 2.6 (0.04 H, s, H-5eq), 3.3 (0.5 H, s, H-7ax), 3.8 (0.14 H, s, H-7 eq); 100-MHz ^1H NMR (labeled **4**, CF_3CO_2H) δ 1.1 (3 H, s, CH_3), 2.0 (0.7 H, s, H-5ax), 2.6 (0.06 H, s, H-5eq), 3.2 (0.49 H, s, H-7ax), 3.8 (0.18 H, s, H-7eq). Neither **3** nor **4** gives a suitable mass spectrum. However, it is possible to carry out deuterium analysis by examining the GCMS of the disilyl derivative of the 6-methyl-5-deazatetrahydropterin (**2**) formed from their reduction by using the same conditions as employed for the analysis of deuterated **2**. GCMS (**2** from labeled **4**) m/e 316, 2.0%; 315, 7.9%; 314, 25.9%; 313, 72.2%; 312, 100.0%; 311, 24.1%; 310, 3.9%; 309, 1.3%; 308, <1%. The pattern is identical within the limits of experimental error with that of the deuterio-**2** starting material (i.e., 28% tetra-, 57% tri-, and 15% di-deuterio).

Reduction of the Bromo Adduct (3). (a) Preparative Reactions. A solution of 2.3 mg of the bromo adduct in 3 mL of H_2O was added to a solution of 10.3 mg of NADH (Sigma) in 8 mL of 0.005 M ammonium bicarbonate (pH 8.0). The reaction mixture was allowed to stand at room temperature until all of **3** was consumed (the reaction was followed by UV). The reaction mixture was then placed on a 8.5 × 0.5 cm column of Dowex 1-X4, 100–120 mesh in the carbonate form and eluted with water with 1.5-mL fractions being collected. Following the elution of a small quantity of the hydroxy adduct (fractions 2–13), **2** was collected in fractions 14–36. The latter fractions were combined and lyophilized to afford a 90% yield of **2** (determined spectrophotometrically). The product was analyzed by GCMS.

The identical procedure was employed in reactions by using sodium borodeuteride, borohydride, or in deuterium oxide as solvent.

(b) **Kinetics.** The disappearance of **3** was followed spectrophotometrically at 340 nm. Reactions were initiated by adding the appropriate volume of a stock solution of **3** (0.429 mg in 1 mL of H_2O) to a cuvette containing NADH and 0.1 M Tris buffer (pH 8.09, μ = 0.1, 25 °C) to give a final volume of 1 mL. NADH was used at concentrations of 1.14×10^{-4} to 2.35×10^{-4} M and **3** at 0.110×10^{-4} to 1.10×10^{-4} M. A plot of $\log [([\mathbf{3}]_i - [\mathbf{3}]_t)/([\mathbf{3}]_t - [NADH]_i)]$ vs. time was linear for each experiment giving a slope corresponding to a second-order rate constant of 3.9 ± 0.2 M^{-1} s^{-1}. The rate was also shown to be independent of buffer concentration.

Bromination with (3). A solution containing 3.91 mg of **3** and ca. 5 mg of 1,3-dimethoxybenzene in 10 mL of 0.05 M hydrobromic acid was stirred at room temperature. The reaction was followed by LC and was complete (all **3** consumed) after 24 h giving a 70% yield of 1-bromo-2,4-dimethoxybenzene (determined by integration of the LC or GC traces) based on **3**. The identity of the product was confirmed by GCMS (column a, 140 °C) and by comparison with an authentic sample.[36]

The reaction was also conducted by using 0.01 M HCl as solvent to give a 15% yield of 1-bromo-2,3-dimethoxybenzene. No bromination was observed by using 0.05 M trifluoroacetic acid, 0.05 M acetate (pH 5), or 0.02 M ammonium bicarbonate (pH 8) as solvent. The yield of bromination (in HBr) could be improved to ≳90% by using the substrate in tenfold excess.

4a-Hydroxy-6-methyl-5-deazatetrahydropterin (5). A solution of 1 g of **2** (monotrifluoroacetate salt) in 10 mL of formic acid was treated with 0.7 mL of 30% hydrogen peroxide at 10 °C. The mixture was stirred at 10 °C until the reaction appeared complete by LC (~2 h) when the solvent was removed under high vacuum (at <25 °C).

The crude product, which was shown by LC and NMR to be a mixture of the cis and trans isomers in the ratio ~4:6, was recrystallized five times from methanol-ether at room temperature to afford 250 mg (30%) of the pure (>95%) trans isomer as the formate salt: mp 242–244 °C; ^{13}C NMR (CF_3CO_2H) δ 18.0 (CH_3), 23.6 (C-6), 34.5 (C-5), 51.3 (C-7), 67.1 (C-4a), 157.8 (C-2), 169.5 (C-4 or C-8a), 170.8 (C-4 or C-8a); ^{13}C NMR Me_2SO δ 18.3 (CH_3), 22.3 (C-6), 33.8 (C-5), 47.9 (C-7), 62.9 (C-4a), 159.8 (C-2), 171.3 (C-8a and C-4). Anal. Calcd for $C_9H_{14}N_4O_4$: C, 44.6; H, 5.8; N, 23.1. Found: C, 44.7; H, 5.81; N, 22.9. Trituration of the oily recrystallization residue with ether afforded the cis isomer in ~60% purity (the major contaminants are the trans isomer and the rearrangement product). ^{13}C NMR (HCO_2H) δ 19.3 (CH_3), 26.7 (C-6), 36.2 (C-5), 46.9 (C-7), 66.1 (C-4a), 157.7 (C-2), 167.3 (C-4 or C-8a), 171.8 (C-4 or C-8a); ^{13}C NMR (Me_2SO) δ 19.8 (CH_3), 26.4 (C-6), 36.0 (C-5), 45.1 (C-7), 63.4 (C-4a), 161.0 (C-2), 172.5 (C-8a or C-4), 172.7 (C-8a or C-4).

2-Imino-6′-methylspiro[oxazolidine-5,3′-piperidine]2′-one-4-(*O*-methyloxime) (9a). *trans*-**5** (50 mg) was added to 1 mL of a 2 M solution of methoxyamine (pH 4.8) and the mixture stirred at room temperature for 16 h. The precipitate was collected by centrifugation and washed with water (4×) and acetonitrile (2×). Recrystallization of the crude product from methanol gave 15 mg (39%) of **9a**: mp 264–266 °C. High resolution MS Calcd for $C_9H_{14}N_4O_3$: 226.1065. Found: 226.1071; 100-MHz ^{1}H NMR (D_2O) δ 1.0 (d, J = 6.1 Hz, CH_3), 1.5–2.2 (3 H, complex), 2.7–3.2 (2 H, complex), 3.5 (3 H, s, OCH_3).

2-Imino-6′-methylspiro[oxazolidine-5,3′-piperidine]2′-one-4-semicarbazone (9b). *trans*-**5** (100 mg) was added to 1 mL of a 2 M solution of semicarbazide (pH 4.8). After 16 h or when LC showed all of **5** had been consumed, the product was collected by centrifugation, washed successively with water (4×) and acetonitrile (2×), and dried. Recrystallization of this product from H_2O gave 60 mg (57%) of **9b**: mp 242–244 °C; MS *m/e* 254 (M^+); NMR (D_2O) δ 1.1 (3 H, d, J = 6.1 Hz, CH_3), 1.3–3.0 (5 H, complex). Anal. Calcd for $C_9H_{14}N_6O_3 \cdot H_2O$: C, 39.7; H, 5.9; N, 30.9. Found: C, 39.6; H, 5.7; N, 30.8.

X-Ray Analysis. Crystals of **9b** were grown from water and contain one water of crystallization per molecule of **9b**. The crystals are monoclinic and belong to the space group $P2_1/C$ with a = 8.36 (1), b = 12.34 (1), c = 12.24 (1) Å, β = 101.34(6)°, and Z = 4. Three-dimensional intensity data were collected with an Enraf-Nonius CAD-4 single crystal diffractometer with Cu Kα radiation and were refined using the Enraf-Nonius SDP program set (1978). The structure was solved by using direct methods. The anisotropic least-squares refinement, with fixed isotropic thermal parameters for the hydrogen atoms, gave a final R index of 8.3% by using 742 observed (nonzero) reflections.

Some disorder accounting for a high R index may arise from the existence of two tautomeric forms of **9b** (with a 2-imino group or a 2,3-double bond in the oxazolidine ring) in the crystal lattice. Nevertheless there is no ambiguity with regard to the skeleton of **9b**. A complete listing of the atomic parameters, bond angles, and bond lengths may be obtained from the authors.

Reactions in $H_2^{18}O$. The reactions to give **9a** and **9b** were conducted as described above but by using $H_2^{18}O$ (7–80%)[37] in the solvent. Analysis of the product both by MS and GCMS (of the disilyl derivative) showed no ^{18}O incorporation. Compound **5** was dried immediately before use to ensure that it was not hydrated (confirmed by elemental analysis). In addition in several experiments, **5** was incubated with acid (CF_3CO_2H or aqueous HCl, pH 1.0) prior to the addition of the semicarbazide or methoxyamine.

4a-Hydroperoxy-6-methyl-5-deazatetrahydropterin. The irradiation apparatus was constructed according to Denny and Nickon[25] and used a Sylvania 600-W high silica tungsten-halogen projection lamp as the light source. A solution of 100 mg of **2** (monotrifluoroacetate salt) in 150 mL of methanol containing 1 g of suspended immobilized Rose Bengal (Polysciences) was photooxygenated for 2 h, or until LC or UV showed **2** to be completely consumed. The mixture was then filtered and the solvent evaporated to leave a pale-yellow oil which was triturated with anhydrous ether to give 85 mg (76%) of **6** as a white crystalline solid.

The purity of the peroxide was gauged by adding a sample of **6** (to give ~10^{-4} M solution) to 0.1 M potassium iodide in methanol and measuring the I_3^- formed spectrophotometrically (ϵ_{349} ~ 29 000).[27,28] Typical values were 50–80%. The LC analysis showed that the **5** formed upon the reduction of **6** was a mixture of cis and trans isomers in the ratio ~4:6.

Oxidation of Thioxane. The oxidation was initiated by the addition of 0.2–1.0 mL of 0.5 M thioxane in methanol to a solution of the hydroperoxide **6** (~10^{-5} M) in 10 mL of methanol. Aliquots of the reaction mixture were withdrawn and their peroxide content analyzed spectrophotometrically. A plot of log [**6**] vs. time was a straight line with slope proportional to the concentration of thioxane. The derived second-order rate constant is given in Table III. The identity of the oxidation product (thioxane sulfoxide) was proved by GCMS and by comparison with an authentic sample (prepared by the reaction of thioxane with hydrogen peroxide in aqueous acetone[38]).

Assays with Phenylalanine Hydroxylase. A sample of phenylalanine hydroxylase (EC 1.14.16.1) purified from rat liver was supplied by Dr. S. Kaufman. The assay solution of 50 mM potassium phosphate (pH 6.8), 2 mM phenylalanine, 0.03 mM **2–5**, and 4 × 10^{-3} units of phenylalanine hydroxylase (sp act. 0.14 μmol min^{-1} mg^{-1}) in a final assay vol of 1 mL was incubated at 25 °C. The decomposition of the 4a adducts was observed at 300 nm; the rates were indistinguishable from those seen in the absence of the enzyme. After 10 min, 20 μL of 1.5 mM 6,7-dimethyltetrahydropterin[39] was added and the oxidation of the cofactor observed at 325 nm.[29] The initial enzyme rates are shown in Table IV. The assay solutions were saved and the tyrosine content was determined by fluorescence measurements of the nitrosonaphthol derivative.[32,33] The yield of tyrosine in an assay to which no 4a adduct was added was 2.8 μg. The relative yield of tyrosine observed in the other assays is shown in Table IV. In addition to the components mentioned above, several assays contained 850 units of catalase (Sigma, EC 1.11.1.6) (see Table IV).

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References and Notes

(1) The IUPAC nomenclature is 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidine. Preliminary accounts of this work have been published: (a) G. Moad, C. L. Luthy, and S. J. Benkovic, *Tetrahedron Lett.*, 2271 (1978); (b) G. Moad, C. L. Luthy, and S. J. Benkovic, *Chem. Biol. Pteridines, Proc. Int. 6th, Symp.*, 55 (1979).
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Total Synthesis of Vernolepin. 2.[1] Stereocontrolled Synthesis of (±)-Vernolepin

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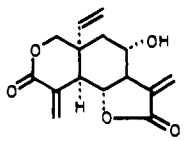
Abstract: The stereocontrolled total synthesis of the bis(α-methylene)lactonic sesquiterpene, vernolepin (**1**), has been achieved. This tumor inhibitor was synthesized in 11 steps starting from the cyclopropane ketone (**3**) as our key synthetic intermediate. This total synthesis has been constructed by a new synthetic strategy based on conformational analyses of the *exo*-olefinic system as IV. The key steps of the synthesis are (i) [2,3]-sigmatropic rearrangement of *p*-anisyl sulfoxide (**8** → **9**) and (ii) intramolecular conjugate reduction with sodium cyanoborohydride in HMPA followed by hydride exchange with diborane.

Vernolepin (**1**) and its congener vernomenin (**2**) have been isolated from *Vernonia hymenolepis* and shown to have sig-



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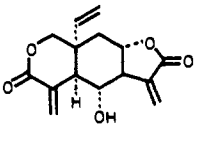
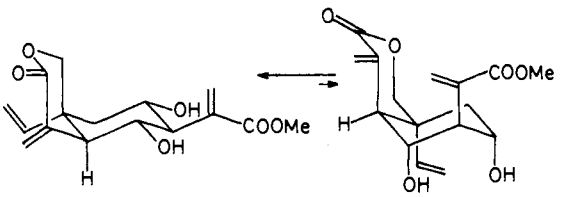


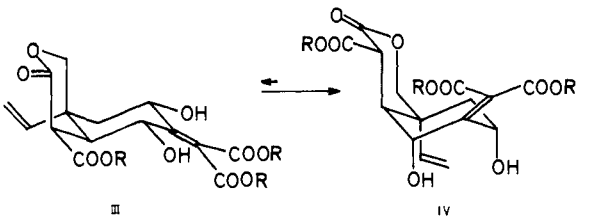
2

nificant in vitro cytotoxicity (KB) and in vivo tumor inhibitory activity against Walker intramuscular carcinosarcoma in rats.[2] Multifaced synthetic attack toward this complex natural product has led to the total syntheses by Grieco,[3] Danishefsky,[4] and ourselves,[5] and a formal total synthesis by Schlessinger[6] and many other valuable synthetic approaches.[7] This full paper, part 2, deals with accomplishment of our total synthesis of **1** from the key synthetic intermediate **3**,[8] the preparation of which has recently been reported in part 1[9] with experimental details.

Conformational analyses of vernolepin prototypes (I to IV) suggest the methodology of functionalization to our key synthetic intermediate (**3**) as shown in Scheme I. The following is our synthetic strategy principally based on the conformational analysis of the B ring of **1**. In the two possible conformers of the acidic methanolysate of **1**, it should exclusively exist in the form of the conformer I rather than II. However, the latter one, the thermodynamically unstable form, contains promising axial bondings to be introduced into the cyclohexanone ring of the precursor (**3**). The fixation of the conformation into the currently interesting type II could be ensured by taking another account for thermodynamic stability, i.e., steric interference in the *exo*-olefin system like III and IV. In this case, equilibration should occur largely into IV as the type II, since in III



I II



III IV

the spacial repulsion between the two hydroxyl groups and two carboalkoxy groups in the planarity would be much greater than the 1,3-diaxial interaction in the conformer IV.[10] Its two axial hydroxyl groups could further assist the stereospecific reduction of the exo-double bond by coordinating with hydride reagent for the hydride to attack from the α side. Introduction of their hydroxyl groups into axial orientation should thus be achieved after condensation of malonyl residue to the key compound (**3**). These conformational analysis and resulting strategy led us to succeed in the stereocontrolled total synthesis (Scheme I) which involves no separation process of stereoisomers at all.

Knoevenagel Condensation of Malonates with Key Compound. The first step for the total synthesis starting from **3** is Knoevenagel condensation, which was first achieved on **11**, as

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