Synthesis and Study of a 6-Amino-5-oxo-3*H*,5*H*-uracil and Derivatives. The Structure of an Intermediate Proposed in Mechanisms of Flavin and Pterin Oxygenases[1]

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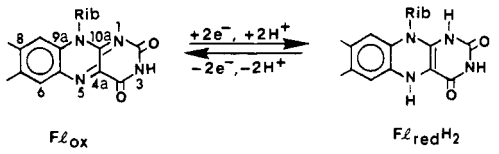
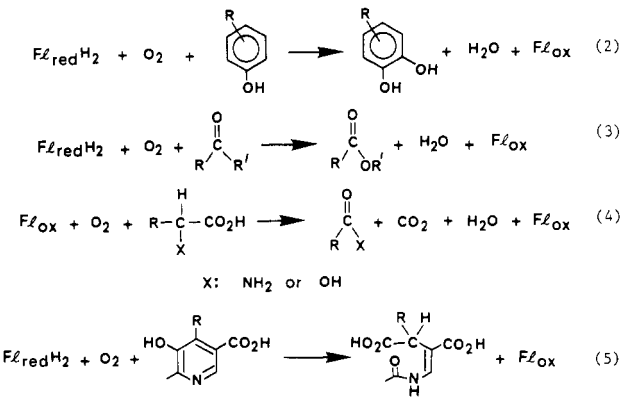
Abstract: The synthesis of 6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (**12b**) has been carried out by peracid oxidation of the N^5-quaternized 3-methyllumiflavin, **10b** (Scheme V), and the preparation of the 5-diazo-3*H*,5*H*-uracils (**4a–c**) and 5-oxo-3*H*,5*H*-uracil hydrazones (**5a–c**) has been carried out as shown in Scheme IV. The structures of these compounds have been firmly established by ^{15}N and ^{13}C NMR as well as by other spectral methods. The 6-amino-5-oxo-3*H*,5*H*-uracil (**12b**) exists in aqueous solution below pH 9 mainly in its unhydrated form (unlike the analogous alloxane which once exposed to H_2O exists as a C^5-covalent hydrate). In methanol **12b** forms an isolable C^5-methanol adduct. Prolonged methanolysis and hydrolysis of **12b** yields *N*,*N*′,*N*′,4,5-pentamethyl-*o*-phenylenediamine (**1c**), establishing that nucleophilic addition of the lyate species occurs at C^6. The pH dependence of the hydrolysis of **12b** has been investigated, and from the determined dependence of the first-order solvolysis constants upon acidity, reaction paths and mechanisms have been suggested. The hydrolytic properties of **12b** have been compared to those of alloxane and flavins. Dithionite and hydrazine reduction of **12b** yields the corresponding 6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-5-hydroxy-3-methyluracil (**13b**), which is easily reoxidized by O_2 in CH_3CN to **12b** in a reaction first order in **13b**. In aqueous solutions **13b** undergoes an autocatalytic oxidation to **12b** reminiscent of dihydroflavin and dialuric acid. The pH dependence of the reduction of **12b** by hydrazine has been investigated. The reaction has been shown to involve the formation of a covalent adduct of hydrazine and **12b**. Further, the adduct is not the hydrazone of **12b** nor is it in equilibrium with **12b** hydrazone (**5c**). The mechanism, which does not involve general or specific catalysis, is discussed. In CH_3CN solvent the electrochemical reduction of **12b** compares favorably to that of flavins. The electrochemical reduction of **12b** occurs in two steps. The first one-electron transfer is quasi-reversible (E_{m_1} = 0.42 V NHE) while the second electron transfer appears chemically reversible but electrochemically nonreversible (E_{m_2} approximates −1.1 V NHE). The IR spectral properties of the related 5-diazo-3*H*,5*H*-uracils (**4a–c**) and their reactivity are interpreted in terms of the most important canonical forms which contribute to their valence bond structures. It is concluded that the diazo carbonyl form (eq 14, A) is more important than the more charge delocalized diazonium enolate (eq 14, B) and that the oxadiazolo structure (V) can be disregarded. The 5-oxo-3*H*,5*H*-uracil 5-hydrazone (**5c**) cannot be prepared by reaction of hydrazine with **12b** due to hydrazine reduction of **12b** to **13b**. Once prepared **5c** cannot be hydrolyzed to **12b** without destruction of the pyrimidine ring. The hydrazones may be considered as isoelectronic and isosteric with corresponding resonance forms of proposed flavin- and pterin-derived carbonyl oxide intermediates in enzymatic monooxygenation reactions. By ^{15}N NMR, their structures have been shown to be best represented by the resonance structures A and B in eq 16; diazene structures like VIIa and VIIb can be neglected. Comparison of the UV/vis spectra of compound II of phenolic hydroxlases to that of **12b** establishes that the enzyme-bound intermediate of these flavomonooxygenases does not possess the electronic structure represented by the 6-amino-5-oxo-3*H*,5*H*-uracil **12b** as proposed by others.

Mono- and dioxygenases catalyze the insertion of one and two atoms of molecular dioxygen, respectively, into their substrates.[2] A substantial number of monooxygenases use flavin cofactors, whose isoalloxazine nuclei undergo reversible oxidoreduction (cf. eq 1) during the catalytic cycle.[3] Most of these flavoenzymes



(1)

are of bacterial origin and monooxygenate either phenols, carbonyl compounds, or α-hydroxy and α-amino acids, according to the stoichiometric equations 2–4. The two known flavin dioxygenases catalyze the dioxygenation and reduction of pyridine derivatives in bacterial metabolism of pyridoxine (eq 5)[4] and the oxygenation



of two molecules of 2-nitropropane.[5] Compared to the wealth of rather specific bacterial enzymes, the role of flavin monooxygenases in higher animals seems to be limited in type. Only one, the broadly specific microsomal flavin dependent monooxygenase, is known.[6] It oxygenates amines, organic sulfides, and thiols (eq 6) which then can undergo further reactions. In

$$Fl_{red}H_2 + O_2 + NR_3 \rightarrow {}^{-}ON^{+}R_3 + H_2O + Fl_{ox} \quad (6a)$$

(1) Part of this publication has already been communicated in preliminary form: Wessiak, A.; Bruice, T. C. *J. Am. Chem. Soc.* **1981**, *103*, 6996–6998.

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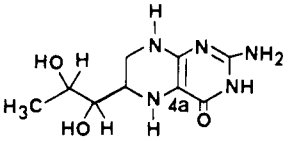
$$Fl_{red}H_2 + O_2 + SR_2 \rightarrow {}^{-}OS^{+}R_2 + H_2O + Fl_{ox} \quad (6b)$$

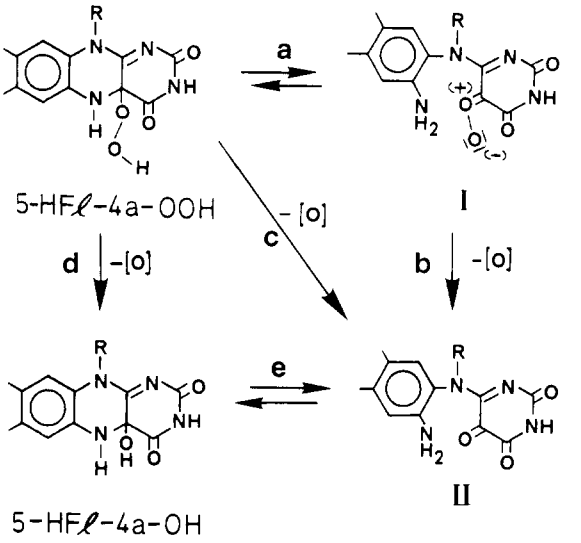
mammals phenylalanine is hydroxylated by an enzyme system using tetrahydrobiopterine (PtH_4; cf. formula) as a cofactor.[7,3b] Deficiencies in this enzyme result in the severe metabolic disorder phenylketonuria.[8,7]



PtH_4

Spectral evidence (UV/vis) for an enzyme-bound dihydroflavin ($Fl_{red}H_2$) oxygen compound has been found for *p*-hydroxybenzoate hydroxylase and other phenol hydroxylases,[3,9] bacterial luciferase,[10] and microsomal flavin dependent monooxygenase.[6a] The spectra of the enzyme-bound intermediates compare well with that for free and enzyme-bound N^5-blocked flavin 4a-pseudobases,[11a] and by use of ^{13}C NMR spectroscopy in the case of bacterial luciferase, it was possible to show that dioxygen was bound to the C(4a) position of the dihydroflavin cofactor.[11b] The synthesis of N-(5)-blocked (i.e., alkylated) 4a,5-dihydro-4a-hydroperoxyflavins in this laboratory[12] established the virtual identity of their spectra with those of enzyme-bound intermediates and allowed the investigation of mono-[13] and dioxygen[14] transfer from synthetic 4a-hydroperoxyflavins to substrates in model reactions. It is now accepted that the first intermediate observed after the reaction of enzyme-bound FlH_2 with dioxygen is a 4a,5-dihydro-4a-hydroperoxyflavin (cf. 5-HFl-4a-OOH in Scheme I). Circumstantial evidence for the formation of an oxygen adduct of tetrahydrobiopterin was provided by the UV/vis spectrum of an intermediate formed as a result of the action of phenylalanine hydroxylase.[15] The structure of a 4a,5-hydrate of quinoid 6,7-dihydrobiopterin (analogous to 5-HFl-4a-OH in Scheme I) was tentatively assigned, which was later confirmed by product studies with modified cofactors (5,6-diaminopyrimidines)[16] and by data

Scheme I



from both chemical model (5-deazapterin 4a-hydroxides and hydroperoxides) and enzyme studies.[17,7b] No data are available for an enzyme bound tetrahydrobiopterin hydroperoxide.

Hamilton has proposed[18] that, prior to "oxene" transfer, flavin and pteridine hydroperoxides undergo activation by means of ring opening (Scheme I, equilibrium a) leading to a carbonyl oxide (I), which can be regarded as a vinylogous ozone or as its valence isomer, a primary ozonide of an acetylene compound.[19] According to the proposal of Hamilton, transfer of monooxygen from the intermediate carbonyl oxide to substrate leaves the "flavin residual" as a *6-amino-5-oxo-3H,5H-uracil* (compound II, Scheme I), which can recycle to oxidized flavin (Fl_{ox}) *via* the intermediacy of 5-HFl-4a-OH (equilibrium e).

Monooxygen transfer from synthetic N(5)-blocked 4a-hydroperoxyflavins (5-RFl-4a-OOH) to amines and alkyl sulfides is strictly first order with respect to both substrate and hydroperoxide. No evidence could be obtained for an intermediate formed by 4a–5 ring opening of the flavin hydroperoxide.[13] The findings obtained with the synthetic flavin hydroperoxides are corroborated in studies with the enzyme-bound flavin 4a-hydroperoxide of the microsomal flavin dependent monooxygenase.[20] Only reaction d of Scheme I (5HFl-4a-OOH → 5HFl-4a-OH) is necessary to explain the data available from enzymology and model chemistry for flavomonooxygenase N- and S-oxygenations. Phenol hydroxylases such as *p*-hydroxybenzoate hydroxylase,[3,9] on the other hand, do exhibit evidence for the presence of an intermediate between the 5-HFl-4a-OOH and the 5-HFl-4a-OH species. Massey et al.[21] have proposed that monooxygen transfer with *p*-hydroxybenzoate hydroxylase occurs by a (in this case concerted) 4a,5 ring opening (Scheme I, reaction c). The spectrum of the enzyme-bound intermediate ($\lambda_{max} \sim 390$–420 nm, $\epsilon = 12000$–16000 M^{-1} cm^{-1}) was assigned to the same 6-amino-5-oxo-3*H*,5*H*-uracil (compound II, Scheme I) as proposed by Hamilton. The same 6-amino-5-oxo-3*H*,5*H*-uracil has been proposed by Khishore and Snell[4] to be formed in the mechanism of a flavodioxygenase enzyme. The expected redox properties of such a species have been incorporated into a recently proposed mechanism[22] for the copper-dependent

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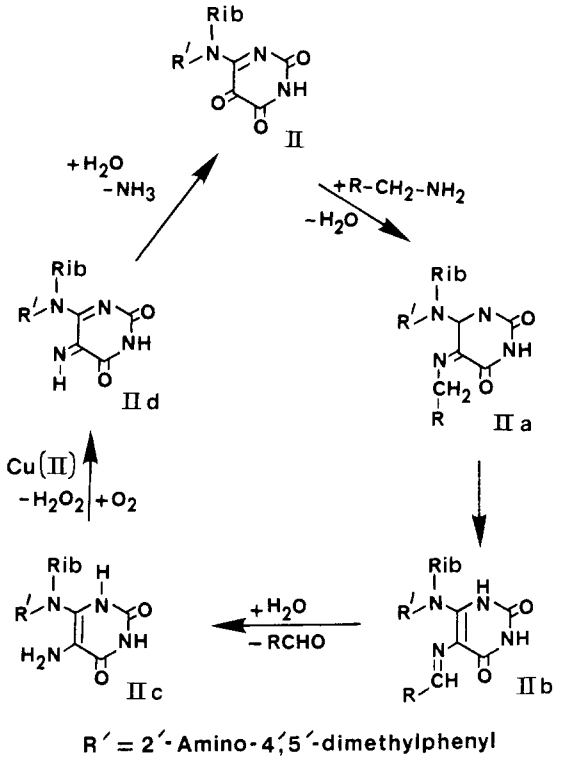
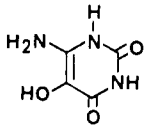
Scheme II



plasma diamine oxidase by Hamilton (cf. Scheme II). This enzyme catalyzes the oxidative deamination of primary amines to aldehydes. It contains, apart from copper, an unknown organic cofactor. The suggestion of Hamilton is that the unknown cofactor is the same 6-amino-5-oxo-3*H*,5*H*-uracil previously proposed to be found as an intermediate in the mechanism of flavin monooxygenases and dioxygenase. Ayling and Bailey[16a-f] have also interpreted their results with modified cofactors as compatible with an oxygen transfer mechanism for phenylalanine hydroxylase whereby the 4a-hydroperoxybiopterin undergoes 4a–5 ring scission to yield a carbonyl oxide which, as in the proposals of Hamilton and Massey, transfers an "oxene" to substrate and yields a 6-amino-5-oxo-3*H*,5*H*-uracil.

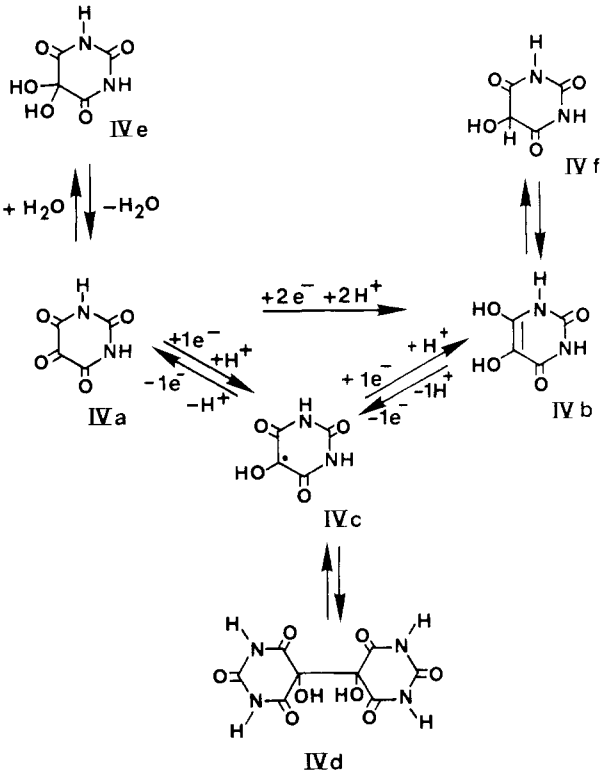
All discussions of ring opening mechanisms which involve flavin mono- and dioxygenase enzymes and the pteridine enzyme phenylalanine hydroxylase have suffered from the fact that 6-amino-5-oxo-3*H*,5*H*-uracils are unknown as a class of compounds.[23] The natural product convicine [6-amino-5-hydroxyuracil (III), conjugated as a glucoside in fava beans[24]] represents



III

two-electron-reduced 6-amino-5-oxo-3*H*,5*H*-uracil. Oxidation of III in aqueous solution does not lead to the monomeric 6-amino-5-oxo-3*H*,5*H*-uracil but to the dimeric product alloxantin[24a] (IVd in Scheme III). This demonstrates the relationship of 6-amino-5-oxo-3*H*,5*H*-uracils with alloxan. The complete redox system of alloxan (IVa or the hydrate, IVe) (Scheme III) consists of dialuric acid (tautomers IVb or IVf), its radical (IVc), and the dimer alloxantin (IVd).[25]

Scheme III



Given the assigned roles which a 6-amino-5-oxo-3*H*,5*H*-uracil plays in the interpretation of the reaction mechanism of flavin- and biopterin-dependent monooxygenation reactions, and in the light of its postulation as a new cofactor, it seemed to us most unsatisfactory that no reliable chemical model exists to assist in the interpretation of the enzymological data. Apart from their use concerning the interpretation of enzyme mechanisms, derivatives of 6-amino-5-oxo-3*H*,5*H*-uracil may be of some interest as cytotoxic agents of the convicine,[24] divicine,[26] and alloxan–dialuric acid[27] type, as different substituents could provide them with different specificities. We describe in this publication the synthesis, characterization, and reactions of a 6-amino-5-oxo-3*H*,5*H*-uracil (a 4a,5 ring opened flavin **12b**) and related derivatives. As a portion of the study there is described the electrochemistry and solution chemistry of **12b** and its dihydro derivative **13b** and the kinetics for the hydrazine reduction of **12b** to **13b**.

Experimental Section

Methods. Melting points were not corrected. Thin-layer chromatography was done on precoated silica gel sheets with fluorescence indicator (EM reagents). The solvent systems used were made by mixing 14 parts of either benzene (A), chloroform (B), toluene (D), or dichloromethane (E) with methyl ethyl ketone (2 parts)/methanol (1 part). Combining 9 parts of the solvent systems A, B, D, or E with 1 part of acetic acid gave the solvent systems A^+, B^+, D^+, and E^+. Adding 3 parts of acetic acid to 7 parts of acetonitrile gave solvent system C. All the systems containing acid were mixed immediately before use and discarded after 1 day; solvent systems containing no acid could be stored.

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Column chromatography was performed on silica gel 60, particle size 0.063–0.200 mm (EM reagents). Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN. Infrared spectra of solids were taken from potassium bromide (KBr) pellets on a Perkin-Elmer 137 sodium chloride spectrophotometer. Ultraviolet/visible spectra were taken on a Cary 15, Cary 118c, and Perkin-Elmer λ 3 spectrophotometers which were also used for the kinetic runs. Proton nuclear magnetic resonance spectra were taken on a Varian T-60 apparatus with tetramethylsilane (Me_4Si) as the internal standard. Carbon-13 nuclear magnetic resonance spectra were taken on a Bruker 360-MHz instrument with Me_4Si as the reference. ^{15}N nuclear magnetic resonance spectra of a 0.46 M solution of **5b** in dimethyl-d_6 sulfoxide were taken on a Bruker WM-500 NMR spectrometer (Southern California Regional NMR Center) operating at 50.65 MHz. The chemical shifts are reported in ppm upfield from 1 M $^2HNO_3$. The spectra were taken with a 31 250-Hz sweep width, 32K data points, 33° pulse angle, and a 6-s delay. Inverse-gated proton decoupling was used to obtain a proton decoupled spectrum with NOE suppression and gated decoupling was used to obtain a proton coupled spectrum with NOE. Mass spectra were taken in house on a VG Micromass ZAB-2F spectrometer. Analytical pH determinations were done with a Beckman 4500 digital pH meter or a Radiometer pH meter, 26. Radiometer GK-2402C, Beckman 39505 and 39822, and Methrom E-125 combination electrodes were used. All measurements were done at 30 ± 0.2 °C and are accurate within ±0.02 pH unit. Standardizations were done with Mallinckrodt Ar buffer solutions at pH 2.00, 4.01, 6.99, and 9.95, which had been kept in a refrigerator prior to use. All compounds synthesized were dried at room temperature at aspirator vacuum over phosphorus pentoxide (P_2O_5) or potassium hydroxide (KOH) for at least 24 h.

Dimethylamine hydrochloride (1a) and *N*-methylaniline (1b) were obtained from Aldrich.

***N,N',N'*-4,5-Pentamethyl-*o*-phenylenediamine (1c)** was prepared by hydrolysis of *N*-formyl-*N,N',N'*,4,5-pentamethyl-*o*-phenylenediamine[28] in 3 M sulfuric acid: $C_{11}H_{18}N_2$ (M_r 178.27); ^1H NMR ($CDCl_3$) δ 6.83 (s, 1 H) and 6.43 (s, 1 H) [C(3,6)H_2], 2.83 [s, 3 H, C(1)NHCH_3], 2.62 [s, 6 H, C(2)N(CH_3)$_2$], 2.23 (s, 3 H) and 2.17 (s, 3 H) [C(4,5)(CH_3)$_2$]. *N*-Formyl-*N',N',N'*,4,5-pentamethyl-*o*-phenylenediamine[28] was synthesized analogous to the preparation of *N*-formyl-*N,N',N'*-trimethyl-*o*-phenylenediamine by Sekiya et al.[29]

6-Chloro-3-methyl-5-nitrouracil (2)[30] and **6-(dimethylamino)-3-methyl-5-nitrouracil (3a)**[30b] were prepared according to the literature.

3-Methyl-6-(methylphenylamino)-5-nitrouracil (3b) was synthesized according to the literature[31] but by using methanol as a solvent.

6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-nitrouracil (3c). *N,N',N'*,4,5-Pentamethyl-*o*-phenylenediamine (**1c**) (1.44 g; 8.08 mmol) was dissolved in 10 mL of methanol to which were added 1.50 g (7.30 mmol) of 6-chloro-3-methyl-5-nitrouracil (**2**) and 1.5 mL (1.41 g; 11.8 mmol) of *N,N*-dimethylaniline. The solution was stirred overnight. A precipitate, which turned out to be the hydrochloride of the product, was filtered off and washed with methanol and diethyl ether. To the filtrate was added 1 mL of acetic acid (1.05 g; 17.5 mmol), and after evaporation to dryness, the residue was taken up in water and the pH adjusted to 5 with base. The aqueous phase was extracted with chloroform. After being dried over $MgSO_4$, the chloroform phase was evaporated, and the residue was taken up again in a minimum amount of chloroform and purified by chromatography over 100 cm^3 of silica gel (i.d. = 3 cm) with chloroform as the eluent. Evaporation of the chloroform provided a yellow oil, to which diethyl ether was added. After several hours the product was filtered off and dried over KOH: yield 0.92 g (32.8%); mp 184–190 °C. The hydrochloride of **3c** (see above) was taken up in sodium hydrogen carbonate solution, which was brought to a pH of 5 with acetic acid. The free base **3c** was obtained as described above without need of column purification: yield 160 mg (5.7%); $C_{16}H_{21}N_5O_4 \cdot H_2O$ (M_r 365.38); mp 189–191 °C dec; TLC R_f = 0.31 (D), R_f = 0.85 (E); IR (KBr) 1700 [C(4)=O], 1655 [C(2)=O], 1600, 1500 (Ar, NO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.03 (s, 1 H) and 6.93 (s, 1 H) [C(3′,6′)H_2], 3.25 [s, 3 H, C(6)NCH_3], 3.20 [s, 3 H, N(3)CH_3], 2.80 [C(2′)N(CH_3)$_2$], 2.29 (s, 3 H) and 2.23 (s, 3 H) [C(4′,5′)(CH_3)$_2$]. Anal. Calcd: C, 52.59; H, 6.34; N, 19.17. Found: C, 52.75; H, 6.04; N, 19.08.

General Method of Preparation of 5-Diazo-3*H*,5*H*-uracils. One gram of the 6-(disubstituted amino)-5-nitrouracil (**3a–c**) was hydrogenated over 0.1 g of palladium/10% on charcoal (Matheson) in the designated volume of water. After the hydrogenation was completed, an appropriate amount of 6 M sulfuric acid was added to give an aqueous solution 1 M in sulfuric acid. The catalyst was filtered off, and 2.00 g (29 mmol) of sodium nitrite was added. Following stirring for 1 h, the product was isolated by extraction with chloroform. After drying over $CaCl_2$, the solvent was evaporated, giving a crude product that was either used for further synthesis or purified as described.

5-Diazo-6-(dimethylamino)-3-methyl-3*H*,5*H*-uracil (4a). 6-(Dimethylamino)-3-methyl-5-nitrouracil (**3a**) (4.67 mmol) was reduced in 50 mL of water. The product was obtained by continuous extraction: yield 474 mg (53.1%) of crude material, dried over KOH; mp 123–133 °C; good for synthesis. Analytical material was obtained by crystallization from ethyl acetate: mp 140–141 °C dec; $C_7H_9N_5O_2$ (M_r 195.18); TLC R_f = 0.48 (E^+); IR (KBr) 2130 (N_2), 1675 [C(4)=O], 1625 [C(2)=O], 1560 cm^{-1}; UV (acetonitrile) λ_{max} (ε, M^{-1} cm^{-1}) 214 nm (25 500), 281 (16 200); ^1H NMR (Me_2SO-d_6) δ 3.21 [s, 6 H, C(6)N(CH_3)$_2$], 3.10 [s, 3 H, N(3)CH_3]. Anal. Calcd: C, 43.07; H, 4.65; N, 35.39. Found: C, 42.89; H, 4.77; N, 35.19.

5-Diazo-3-methyl-6-(methylphenylamino)-3*H*,5*H*-uracil (4b) was synthesized by previously published methods.[32] 3-Methyl-6-(methylphenylamino)-5-nitrouracil (**3b**) (3.62 mmol) was reduced in 100 mL of water. The chloroform extract was washed with a small volume of water before drying. **4b** of sufficient purity was obtained by washing the suspended residue of the dried chloroform extract in low-boiling petroleum ether: yield 724 mg (77.7%), dried over KOH; mp 178–180 °C dec. Analytical material was obtained by recrystallization from water: $C_{12}H_{11}N_5O_2$ (M_r 257.25); mp 182–183 °C (decomposes to another product which does not melt below 350 °C, lit.[32] mp 185 °C); TLC R_f = 0.60 (B^+); IR (KBr) 2200 (w), 2140 (s), 2110 (w, N_2), 1690 [C(4)=O], 1640 [C(2)=O], 1540, 1520 cm^{-1}; UV (acetonitrile) λ_{max} (ε, M^{-1} cm^{-1}) 204 nm (26 300), 222 sh, 284 (16 000); ^1H NMR ($CDCl_3$) δ 7.57–7.12 (m, 5 H, ArH_5), 3.48 [s, 3 H, N(6)CH_3], 3.24 [s, 3 H, N(3)CH_3]. Anal. Calcd: C, 56.02; H, 4.31; N, 27.23. Found: C, 55.75; H, 4.49; N, 26.90.

5-Diazo-6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-3*H*,5*H*-uracil (4c). 6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-nitrouracil (**3c**) (0.5 g) was reduced in 60 mL of water. Before extraction with chloroform, the solution was partially neutralized by addition of 3.0 g of sodium hydrogen carbonate. The residue obtained from the chloroform extract was taken up in ethyl acetate/chloroform (1/1) and purified by chromatography over a 1-cm column containing 35 mL of silica gel (eluent ethyl acetate/chloroform = 1/1). Evaporation of the eluent gave yellow crystals, which were dried over KOH: yield 520 mg (55.0%); $C_{16}H_{20}N_6O_2$ (M_r 328.37); mp 180–182 °C dec; TLC R_f = 0.77 (E); IR (KBr) 2130 (N_2), 1695 [C(4)=O], 1645 [C(2)=O], 1545 cm^{-1}; UV (acetonitrile) λ_{max} (ε, M^{-1} cm^{-1}) 205 nm (sh), 225 (32 700), 255 (sh), 281 (17 300), 350 (sh); ^1H NMR ($CDCl_3$) δ 6.86 (s, 1 H) and 6.69 (s, 1 H) [C(3′,6′)H_2], 3.50 [s, 3 H, C(6)NCH_3], 3.32 [s, 3 H, N(3)CH_3], 2.72 [s, 6 H, C(2′)N(CH_3)$_2$], 2.30 (s, 3 H) and 2.19 (s, 3 H) [C(4′,5′)(CH_3)$_2$]. Anal. Calcd: C, 58.52; H, 6.14; N, 25.60. Found: C, 58.34; H, 6.29; N, 25.30.

6-(Dimethylamino)-3-methyl-5-oxo-3*H*,5*H*-uracil 5-Hydrazone (5a). 5-Diazo-6-(dimethylamino)-3-methyl-3*H*,5*H*-uracil (**4a**) (200 mg; 1.02 mmol) was dissolved in 20 mL of dry dioxane (molecular sieves) and to this solution there was added 0.50 g (1.91 mmol) of triphenylphosphine (Eastman) dissolved in 5 mL of dioxane. After the solution was stirred for 1 h 210 mg (3.04 mmol) of sodium nitrite was added, the solution cooled in an ice bath, and 6 mL of 1 M sulfuric acid added dropwise. The solution was stirred at room temperature overnight when 75 mL of water was added and the pH adjusted to 6 with sodium hydrogen carbonate. The solution was extracted with chloroform, until no further product was detected in the extract. After being dried over $CaCl_2$, the chloroform was evaporated and the product precipitated from the resultant solution by addition of an excess of diethyl ether. The product was collected by filtration, washed with diethyl ether, and dried over KOH: yield 56 mg (27.5%) of material pure on TLC (solvent system E). Evaporating the mother liquor to dryness and adding diethyl ether to the residue provided a second crop of product [yield 70 mg (34.4%)] contaminated by a small amount of triphenylphosphine oxide: $C_7H_{11}N_5O_2 \cdot {}^1/_8H_2O$ (M_r 199.45); mp 214 °C dec; TLC R_f = 0.42 (E^+); IR (KBr) 3250 (NH_2), 1650 [C(4)=O], 1620 [C(2)=O] cm^{-1}; UV (acetonitrile) λ_{max} (ε, M^{-1} cm^{-1}) 297 nm (12 200); ^1H NMR (Me_2SO-d_6 plus ca. 5% CF_3CO_2H) δ 3.47 (s, 3 H), 3.33 (s, 3 H), 3.20 (s, 3 H); ^1H NMR ($CDCl_3$ plus ca. 10% CH_3CO_2H) δ 3.68 (s, 3 H), 3.54 (s, 3 H), 3.36 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 161.8, 161.6, 155.6 [C(2,4,6)], 116.8 [C(5)], 28.0 [N(3)CH_3], 26.5 [C(6)N(CH_3)$_2$]. Anal. Calcd: C, 42.15; H, 5.68; N, 35.11. Found: C, 42.00; H, 5.55; N, 34.44. Thin-layer chromatography of the analytical sample after several months showed

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the presence of the starting material. It is therefore advisable to synthesize the hydrazone **5a** and its analogues **5b** and **5c** (see below) freshly before use.

3-Methyl-6-(methylphenylamino)-5-oxo-3*H*,5*H*-uracil 5-Hydrazone (5b). 5-Diazo-3-methyl-6-(methylphenylamino)-3*H*,5*H*-uracil (**4b**) (146 mg; 568 μmol) was suspended in 4 mL (4.74 g; 15.26 mmol) of triphenyl phosphite. After ca. 5 h TLC in ethyl acetate showed only ca. 50% conversion into a new spot (R_f = 0.40). Stirring at room temperature was therefore continued for 2 weeks, after which 70 mL of diethyl ether was added. The resultant precipitate was filtered off, washed with diethyl ether, and dried over KOH: yield 33 mg (22%); $C_{12}H_{13}N_5O_2 \cdot {}^1/_4H_2O$ (M_r 261.75); mp 141–146 °C dec; TLC R_f = 0.46 (ethyl acetate); IR (KBr) 3350 and 3100 (NH_2), 1680 [C(4)=O], 1640 [C(2)=O], 1530 (Ar) cm^{-1}; UV (acetonitrile) λ_{max} (ϵ, M^{-1} cm^{-1}) 299 nm (15 000); ^1H NMR ($CDCl_3$) δ 7.50–7.00 (m, 5 H, Ph), 3.57 [s, 3 H, C(6)NCH_3], 3.30 [s, 3 H, N(3)CH_3]; ^{15}N NMR (Me_2SO-d_6) δ 223.3 [N(3)], 201.5 (dd, J = 104 and 92 Hz or 92 and 94 Hz, $N^{\alpha}H_2$), 159.5 [N(1)], −31.1 ($N\beta$). Anal. Calcd: C, 54.60; H, 5.15; N, 26.55. Found: C, 54.73; H, 5.15; N, 25.86. A small IR peak at 2130 cm^{-1} showed the presence of a small amount of the starting material as an impurity. The mother liquor was evaporated, and the residue was dissolved in 2 mL of benzene. Addition of an excess of low-boiling petroleum ether precipitated a further crop of product, which was filtered off and washed with petroleum ether. Drying over P_2O_5 gave a further yield of raw material (71 mg; 47.8%), whose melting point (126–134 °C) and TLC showed the presence of impurities.

3-Methyl-6-(methylphenylamino)-5-(triphenylphosphoranylidenehydrazono)-3*H*,5*H*-uracil Hydrate (5b′). To 550 mg (2.14 mmol) of **4b** dissolved in 50 mL of dry dioxane was added 5.50 g (21.0 mmol) of triphenylphosphine. After 30 min of stirring, the solution was poured into 250 mL of water, to which 5 mL of acetic acid had been added. The resulting suspension was extracted with chloroform, and after being dried over $CaCl_2$, it was evaporated to dryness under oil pump vacuum. The residue was stirred in 100 mL of diethyl ether overnight, and then the crystalline product was filtered off, washed with diethyl ether and dried over KOH: yield 920 mg (82.7%); $C_{30}H_{26}N_5O_2P \cdot H_2O$ (M_r 537.54); mp 158–160 °C dec; TLC R_f = 0.56 (ethyl acetate); IR (KBr) 1680, 1645 [C(2,4)=O], 1525 (Ar) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.95–6.80 (m, 20 H, 4 Ph), 3.30 (s, 3 H) and 3.25 (s, 3 H) [N(3)CH_3 and C(6)NCH_3]. Anal. Calcd: C, 67.03; H, 5.25; N, 13.03; P, 5.76. Found: C, 66.46; H, 5.28; N, 12.82; P, 5.77.

6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil 5-Hydrazone (5c). 5-Diazo-6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-3*H*,5*H*-uracil (**4c**) (186 mg, 566 μmol) was dissolved in 20 mL of dry dioxane (molecular sieve) and 1.00 g (3.81 mmol) of triphenylphosphine was added. The reaction solution, protected from moisture, was stirred for 1 h and poured into 100 mL of water, to which 2.00 mL of acetic acid had been added (giving a pH of about 2–3). After 30 min of stirring, the solution was extracted with chloroform, and the extract was washed with sodium hydrogen carbonate, dried over calcium chloride, and evaporated. To the residue was added a little diethyl ether, followed by an excess of low-boiling petroleum ether. After precipitation was complete (1 h), the suspension was filtered and the precipitate washed with petroleum ether and dried over KOH: yield 32 mg (17.1%); $C_{16}H_{22}N_6O_2$ (M_r 330.38); mp 162–164 °C (dec); IR (KBr) 3350 and 3150 (NH_2), 1680 [C(4)=O], 1640 [C(2)=O], 1530 (Ar) cm^{-1}; UV (acetonitrile) λ_{max} (ϵ, M^{-1} cm^{-1}) 232 nm (26 000), 260 (sh), 300 (15 800); ^1H NMR ($CDCl_3$) δ 6.70 [s, 2 H, C(3,6)H_2], 3.44 [s, 3 H, C(6)NCH_3], 3.28 [s, 3 H, N(3)CH_3], 2.63 [s, 6 H, C(2)$N(CH_3)_2$], 2.23 (s, 3 H) and 2.15 (s, 3 H) [C(4,5)-$(CH_3)_2$]. Anal. Calcd: C, 58.16; H, 6.71; N, 25.44. Found: C, 57.96; H, 6.81; N, 25.19.

5-Diazo-1-methylbarbituric Acid (6). A solution of 3-methyl-6-(methylphenylamino)-5-nitrouracil (**3b**) (0.50 g; 1.81 mmol) in 50 mL of water was hydrogenated over 90 mg of palladium on charcoal when 10 mL of 6 M hydrochloric acid was added and the catalyst removed by filtration. To the filtrate there was added 1 g (14.5 mmol) of sodium nitrite, and after stirring for 30 min the solution was refluxed. From time to time an aliquot was taken, neutralized with sodium hydrogen carbonate, and extracted with chloroform, and the extract was subjected to TLC with solvent system A$^+$. After 70 h, the starting material could not be detected. After being cooled to room temperature, the solution was adjusted to pH 3 with sodium hydrogen carbonate and acetic acid and extracted with chloroform. The extract was dried over $CaCl_2$ and evaporated to ca. 5 mL. The product was precipitated with low-boiling petroleum ether, filtered off, washed with petroleum ether, and dried over P_2O_5: yield 32.38 mg (10.5%); $C_5H_4N_4O_3$ (M_r 168.11); mp 198–208 °C dec; TLC R_f = 0.37 (A$^+$); IR (KBr) 3160 and 3050 (NH), 2180 (N_2), 1745, 1720, 1670 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$ plus 5% Me_2SO-d_6) 3.27 (s, CH_3).

3,9-Dimethylpyrimido[4,5-*b*]indole-2,4(1*H*,3*H*,9*H*)-dione (7) was obtained in analytical purity by irradiation of 27.4 mg (106 μmol) of 5-diazo-3-methyl-6-(methylphenylamino)-3*H*,5*H*-uracil (**4b**) in 30 mL of water–methanol (2:1), as already reported in the literature:[32] yield 20.6 mg (84.8%); $C_{12}H_{11}N_3O_2$ (M_r 229.23), mp above 300 °C; IR (KBr) 1710, 1650 (C=O) cm^{-1}; ^1H NMR (CF_3CO_2H) δ 8.10–7.80 (m, 1 H) and 7.50–7.20 (m, 3 H) (ArH_4), 3.70 (s, 3 H) and 3.57 (s, 3 H) [N-(3,9)$(CH_3)_2$].

3,10-Dimethylisoalloxazine 5-Oxide (8a) and 3,10-Dimethylisoalloxazine (8b). To a solution of 5-diazo-3-methyl-6-(methylphenylamino)-3*H*,5*H*-uracil (**4b**) (73 mg; 284 μmol) in 3 mL of dry dioxane (molecular sieve) was added 1 g (3.81 mmol) of triphenylphosphine dissolved in 2 mL of dioxane. The reaction turned orange immediately. After the mixture was stirred for 90 min, no starting material could be detected on TLC. Sodium nitrite (1 g, 14.5 mmol) was added, followed by 25 mL of 1 M sulfuric acid (dropwise). After being stirred for 2 h, the reaction mixture was extracted with chloroform, which was washed with water and dried over $CaCl_2$. Apart from spots corresponding to triphenylphosphine and its oxidation product triphenylphosphine oxide, a minor product could be detected as a yellow fluorescent spot and was identified as 3,10-dimethylisoalloxazine (**8b**)[33] by comparison with authentical material on TLC in the solvent systems B and E. The main product, having the lowest R_f value, was obtained by chromatography over silica gel with ethyl acetate as the eluent: yield 13.6 mg (18.5%); mp above 400 °C; IR (KBr) 1690 [C(4)=O], 1660 [C(2)=O] cm^{-1}; UV (CH_3CN) λ_{max} (ϵ, M^{-1} cm^{-1}) 266 nm (21 900), 338 (6200), 451 (4800). As the main product can be converted to 3,10-dimethylisoalloxazine by treatment with sodium dithionite as described in the literature,[33] its structure can be assumed to be that of 3,10-dimethylisoalloxazine 5-oxide) (**8a**) [$C_{12}H_{10}N_4O_3$ (M_r 258.23)].[33]

3-Methyllumiflavin (9)[33] and 5-acetyl-1,5-dihydro-3-methyllumiflavin (10a)[34] were synthesized according to the literature.

1,5-Dihydro-3,5,5-trimethyllumiflavin (10b) was synthesized after the method of Ghisla et al.,[35] as modified by Dr. Martin Bruestlein of this laboratory. To a continuously chilled suspension of 3-methyllumiflavin (**9**) (2.00 g; 7.40 mmol) in 200 mL of ethanol (95%) there was added (N_2 atmosphere) 4.00 g of ca. 85% dithionite (20 mmol) dissolved in 200 mL of 1 M NaOH. Stirring in the ice bath was continued for 1 h, when 40 mL (424 mmol) of dimethyl sulfate was added dropwise, so that the temperature did not rise above 5 °C. Stirring was continued at room temperature overnight, and then the solution was heated to 50–55 °C for 5 h. After being cooled to room temperature, the reaction was no longer kept under nitrogen and the ethanol evaporated. The remaining aqueous solution was adjusted to pH 7–8 with sodium hydrogen carbonate (noticeable by a color change of the solution from orange-red to blue-green) and extracted with chloroform and the extract dried over $MgSO_4$ and Norite. After evaporation of the chloroform, the residue was dried over P_2O_5: yield 2.3 g (103.5%); mp 195–200 °C dec. A material pure enough for synthetic purposes was obtained by dissolving the crude product in chloroform–ethyl acetate (8:2) (ca. 140 mL/g) and putting it on a silica gel column (ca. 70 g/g, i.d. = ca. 2 cm). It was washed with chloroform/ethyl acetate (8/2) until all the 3-methyllumiflavin and other impurities had been removed and the product eluted with methanol. Evaporation of methanol and drying gave a yield of ca. 77%; mp 216–220 °C. Analytical material was obtained by applying the crude material [ca. 800 mg/5–10 mL of $CHCl_3$/methanol (9.5/0.5)] to a 1-cm column containing 20 mL of silica gel and eluting with chloroform–methanol (9.5–0.5) which first removes lumiflavin and other impurities (along with a portion of the product). The pure product then emerges. After evaporation of the eluent, the residue was taken up in a small amount of chloroform and precipitated with diethyl ether: mp 230–232 °C dec (lit.[35] mp 231–234 °C). Previous workers[35] report that **10b** decomposes slowly to a yellow product when left in an oxygen atmosphere. We did not observe this reaction with our analytical sample, which was colorless after more than 1 year: $C_{16}H_{20}N_4O_2$ (M_r 300.35); ^{13}C NMR [$CDCl_3/CD_3OD$ (1/1)] δ 159.2, 157.0, 149.0 [C(2,4,10a)], 139.7, 130.7, 129.5, 128.5, 121.2, 115.5 [C(5a,6,7,8,9,9a)], 97.9 [C(4a)], 58.9 [N-(5)$(CH_3)_2$], 28.2, 26.1 [N(3,10)$(CH_3)_2$], 18.0, 17.3 [C(7,8)$(CH_3)_2$].

5-Acetyl-4a,5-dihydro-4a-hydroxy-3-methyllumiflavin (11a). A solution of 790 mg (2.51 mmol) of 5-acetyl-1,5-dihydro-3-methyllumiflavin (**10a**) in 100 mL of chloroform was added to 1.20 g (ca. 5.8 mmol) of 85% *m*-chloroperbenzoic acid. After the mixture was stirred for 1 h only traces of the starting material could be detected on TLC, and the chlo-

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roform solution was extracted with sodium hydrogen carbonate solution followed by water. After being dried over $MgSO_4$, the chloroform was evaporated. The residue was taken up in a small amount of chloroform and purified by chromatography over ca. 10 mL of charcoal (eluent chloroform). After evaporation of the chloroform, the residue was suspended in diethyl ether over the weekend and the product was filtered off and dried over KOH: yield 160 mg (19%); mp 185–190 °C; TLC R_f = 0.63 (E). The mp did not improve after crystallization from diethyl ether: $C_{16}H_{18}N_4O_4 \cdot {}^4/_3H_2O$ (M_r 354.36); IR (KBr) 3400, 3250 (OH), 1735 [C(4)=O], 1690 and 1660 [C(2)=O and N(5)C=O], 1630 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.20 (s, 1 H) and 7.01 (s, 1 H) [C(6,9)H_2], 3.63 [s, 3 H, N(10)CH_3], 3.36 [s, 3 H, N(3)CH_3], 2.36 [s, 6 H, C(7,8)-$(CH_3)_2$], 2.10 [s, 3 H, C(5)NCOCH_3]; ^{13}C NMR ($CDCl_3$) δ 173.0 [N(5)$COCH_3$], 165.9, 162.1, 155.7 [C(2,4,10a)], 136.5, 133.1, 132.5, 127.5, 124.9, 117.5 [C(5a,6,7,8,9,9a)], 71.0 [C(4a)], 31.9 [N(10)CH_3], 27.6 [N(3)CH_3], 22.5 [N(5)$COCH_3$], 19.6, 19.2 [C(7,8)$(CH_3)_2$]. Anal. Calcd: C, 54.23; H, 5.88; N, 15.81. Found: C, 54.13; H, 5.58; N, 15.58.

6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (12b) (Pyrimidine-2,4,5(3*H*)-trione). Sodium hydrogen carbonate (2.00 g, 23.8 mmol) and 240 mg (1.18 mmol) of 85% *m*-chloroperbenzoic acid were suspended in 100 mL of chloroform. After 45 min, there was then added a solution of 340 mg (1.13 mmol) of 1,5-dihydro-3,5,5-trimethyllumiflavin (**10b**) in 100 mL of chloroform. After 30 min of stirring the resultant suspension was washed quickly with water. After being dried over magnesium sulfate, the chloroform was evaporated. TLC (E) showed that the residue consisted mainly of starting material and product. The latter was separated from the starting material by dissolving the residue again in ca. 5 mL of chloroform and applying it to a 1-cm column of 20 mL of silica gel (eluent chloroform). After evaporation of the eluent, the residue was suspended in diethyl ether, first at room temperature and then in an ice bath, after which the product was filtered off, washed with diethyl ether, and dried over P_2O_5: yield 108 mg (30.2%). $C_{16}H_{20}N_4O_3$ (M_r 316.35); mp 224–226 °C dec; TLC R_f = 0.86 (E); IR (KBr) 1720 [C(5)=O], 1700 [C(4)=O], 1665 [C(2)=O] cm^{-1}; UV (acetonitrile) λ_{max} (ϵ, M^{-1} cm^{-1}) 229 nm (24 600), 245 (sh), 281 (12 900), 342 (7120); ^1H NMR ($CDCl_3$) δ 7.10 (s, 1 H) and 6.87 (s, 1 H) [C(3′,6′)H_2], 3.60 [s, 3 H, C(6)NCH_3], 3.39 [s, 3 H, N(3)CH_3], 2.51 [s, 6 H, C(2′)N$(CH_3)_2$], 2.24 [s, 6 H, C(4′,5′)$(CH_3)_2$]; ^{13}C NMR ($CDCl_3$) δ 162.7, 160.5, 159.7, 154.5 [C(2,4,5,6)], 139.2, 136.4, 136.4, 134.7, 124.9, 123.0 [C(1′,2′,3′,4′,5′,6′)], 43.0 [C(2′)N-$(CH_3)_2$], 38.2 [C(6)NCH_3], 28.5 [N(3)CH_3], 19.6, 19.3 [C(4′,5′)-$(CH_3)_2$]. Anal. Calcd: C, 60.74; H, 6.37; N, 17.71. Found: C, 60.56; H, 6.43; N, 17.53. The starting material which had remained on the column with other impurities was washed down with methanol. After evaporation it was separated from a small amount of silica gel by dissolving in chloroform and filtration. Evaporation of the chloroform gave a yield of 120 mg (35.3%) of crude starting material, which could be used again.

6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-5-hydroxy-3-methyluracil (13b). 6-[[2-(Dimethylamino)-4,5-dimethylphenyl]-methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (**12b**) (34 mg; 0.107 mmol) was dissolved in 30 mL of chloroform and put into a separating funnel. To this was added 0.50 g (2.44 mol) of 85% dithionite dissolved in 5 mL of saturated aqueous sodium chloride solution mixed with 5 mL of 2 M acetate buffer (1/1). After being shaken vigorously for 2 min the phases were allowed to separate and the chloroform solution was filtered through a paper filter for drying and immediately evaporated. The aqueous phase was extracted with 10 mL of chloroform, and the extract was filtered through paper and immediately evaporated. Diethyl ether was added to the combined residues, and after 5 min the product was filtered off, washed with diethyl ether, and dried over P_2O_5: yield 24 mg (70.5%); mp starts to decompose at 180 °C and finally melts between 220 and 230 °C. The IR was identical with that of the analytical sample, which was obtained by recrystallization from ca. 6 mL of anaerobic ethanol. Recrystallization did not improve the melting point behavior: $C_{16}H_{22}N_4O_3$ (M_r 318.37); TLC identical with that of **12b** after autoxidation in acetonitrile (E, F); IR (KBr) 3400 (OH), 3100 (NH), 1695 [C(4)=O], 1660 [C(2)=O], 1595 (Ar) cm^{-1}; UV (CH_3CN) λ_{max} (ϵ, M^{-1} cm^{-1}) 318 nm (12 700); converts to the UV of **12b** with isosbestic points at 211, 289, and 331 nm under an oxygen atmosphere. Anal. Calcd: C, 60.36; H, 6.97; N, 17.60. Found: C, 60.05; H, 6.85; N, 17.42.

6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-5-hydroxy-5-methoxy-3-methyl-3*H*,5*H*-uracil (14b). 6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (**12b**) (35 mg; 0.11 mmol) was stirred in 2 mL of methanol (purged with nitrogen) for 24 h, after which the brown suspension had turned colorless. Thin-layer chromatography (solvent: ethyl acetate) showed two product spots with R_f values of 0.47 and 0.91. The solvent was evaporated at room temperature and taken up in ethyl acetate/chloroform (1/1), and the two products were separated over a 1-cm column of 10 mL of silica gel (eluent ethyl acetate/chloroform, 1/1). The ^1H NMR of the product eluted first (R_f = 0.91) proved to be identical with that of *N,N′,N′*,4,5-pentamethyl-*o*-phenylenediamine (**1c**) after evaporation of the solvent. After collection of the fraction containing the main product, the eluent was evaporated at room temperature, the residue was taken up in a minimum amount of chloroform, and the product with the R_f = 0.47 was precipitated with diethyl ether. After being stirred in an ice bath, it was filtered off, washed with cold diethyl ether, and dried over P_2O_5: yield 14.5 mg (37.8%); $C_{17}H_{24}N_4O_4$ (M_r 398.39); mp 184–189 °C dec; TLC R_f = 0.47 (ethyl acetate); IR (KBr) 3400 (OH), 1755 [C(2)=O], 1730 [C(4)=O], 1580 (Ar) cm^{-1}; UV (methanol) λ_{max} 260 nm (sh), 225 (sh); ^1H NMR ($CDCl_3$) δ 6.87 (s, 1 H) and 6.80 (s, 1 H) [C(3′,6′)H_2], 3.50 [s, 3 H, C(6)NCH_3], 3.38 [s, 3 H, N(3)CH_3], 2.77 [s, 6 H, C(2′)N-$(CH_3)_2$], 2.74 [s, 3 H, C(5)OCH_3], 2.29 (s, 3 H) and 2.22 (s, 3 H) [C(4′,5′)$(CH_3)_2$]; MS (70 eV) *m/e* (relative intensity) 348 (M^+, 7.8), 288 ($M - C_2H_4O_2$, 13). Anal. Calcd: C, 58.60; H, 6.94; N, 16.08. Found: C, 58.47; H, 6.97; N, 15.80.

Kinetics. All kinetic runs were followed by UV/vis spectrometry at 30 ± 0.2 °C. Anaerobic runs were performed in a Cary 15 whose sample compartment was built into a N_2 atmosphere dry box. Repetitive scans were taken on a Cary 118C instrument. The acetonitrile used for stock solutions and for kinetics in acetonitrile was from Burdick and Jackson Laboratories and contained 0.008% water. Trizma base (reagent grade) was obtained from Sigma. All other buffers were made up from analytical reagents obtained from Mallinckrodt. Chloroacetic acid, formic acid, acetic acid, potassium dihydrogen phosphate, Trizma base, and sodium hydrogen carbonate were mixed with sodium hydroxide or hydrochloric acid to give buffer solutions whose respective pH values were within ±1 of the pK_as of the buffers used.[36] For the pH extremes pure hydrochloric acid or sodium hydroxide was used to maintain constant pH. The ionic strength of all solutions was brought to 1 with potassium chloride. The pH of buffer dilution series was adjusted to be within ±0.02 pH unit of the respective pH value. All pH values of solutions used for kinetic runs were determined immediately after the end of the run within the cuvette.

Hydrolysis of 6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (12b). A 5.00 mM stock solution of **12b** in acetonitrile was diluted 100 times (giving 5×10^{-5} M reaction solutions containing 1% acetonitrile) with the appropriate buffer. The spectrophotometer was started at the moment when the stock and buffer solutions were mixed, giving thereby a record of the beginning of the reaction, from which the absorption at t = 0 could be calculated by extrapolation. Mixing time was about 10 s. All reactions were followed for seven to ten half-lives, giving pseudo-first-order kinetics and stable end points. Buffer concentrations were 0.02 and 0.2 M for all measurements. When a difference of more than 5% was obtained between observed rate constants (k_{obsd}) at the two concentrations differing by 1 order of magnitude, three more rate constants were determined between those boundaries at 0.065, 0.11, and 0.155 M buffer concentrations, and the rate constants used for the log k_{ly}–pH profile were obtained by extrapolation to zero buffer concentration.

Reduction of 6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (12b) by Hydrazine. All reactions had to be carried out under nitrogen, as the presence of oxygen in the reaction solutions gave complicated kinetic traces due to autoxidation. Otherwise the reactions were done as described for the hydrolysis. All buffers were made by mixing anaerobic stock solutions of hydrazine hydrochloride (Matheson; recrystallized the following way: 2 g was suspended in 100 mL of 95% aqueous ethanol, which was heated to boiling; water was added carefully to the resulting emulsion, until the second phase disappeared; the solution was then stirred at room temperature overnight, and the recrystallized $N_2H_4 \cdot HCl$ (mp 89–91 °C; lit. mp 89 °C[37]) was filtered off, washed with cold ethanol and diethyl ether, and dried over drierite (yield 50%)) and sodium hydroxide. All reactions were done at pH values within ±1 unit of the ionization constant of hydrazine (pK_a = 8.11[38]), and total concentrations were 0.020, 0.065, 0.110, 0.155, and 0.200 M. The ionic strength of the solutions was brought to 1.0 by addition of appropriate amounts of potassium carbonate.

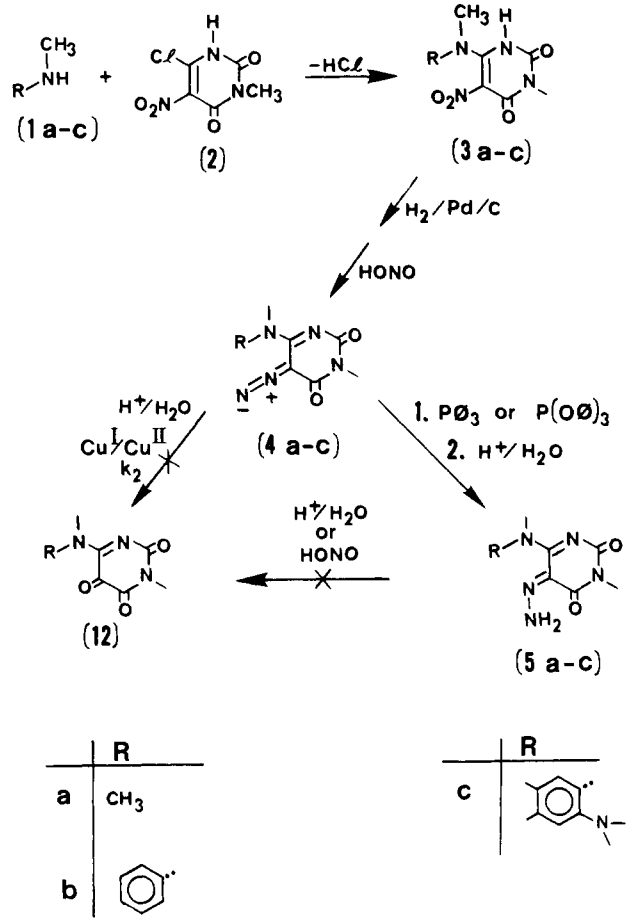
Autoxidation of 6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-5-hydroxy-3-methyluracil (13b) was followed after dilution of a 4.07×10^{-3} M anaerobic acetonitrile stock solution 100 times either with

(36) Perrin, D. D.; Dempsey, B. D. "Buffers for pH and Metal Ion Control"; Chapman: London, 1974.

(37) Weast, R. C.; Astle, M. J. "CRC Handbook of Chemistry and Physics"; CRC Press: Boca Raton, FL, 1980.

(38) (a) Bruice, T. C.; Bruno, J. J.; Chou, W.-S. *J. Am. Chem. Soc.* **1963**, 1659–1669. (b) Condon, F. E.; Reece, R. T.; Shapiro, D. G.; Thakkar, D. C.; Goldstein, T. B. *J. Chem. Soc., Perkin Trans. 2* **1974**, 1112–1121.

Scheme IV



aerobic acetonitrile ($\sim 1 \times 10^{-3}$ M in O_2) or with the aerobic buffers used for the hydrolysis in the manner described for the hydrolysis of **12b**.

Electrochemistry. All electrochemistry was done in acetonitrile solutions which were 0.1 M in tetraethyl ammonium perchlorate (Eastman). The potentials are given vs. the normal hydrogen electrode (NHE). Measurements were performed with a modified PAR Model 174 polarography analyzer (Princeton Applied Instruments).

Working Electrodes. For normal (thick layer) cyclic voltametric experiments a platinum bead electrode with a surface area of approximately 3 mm^2 was employed. The platinum billet and housing for the working electrodes used for thin-layer voltametric[39] experiments were constructed by Prof. A. T. Hubbard of this department. Their volumes were determined to be 4.66 and 4.78 μL by Dr. Timothy Eckert of this laboratory. A platinum wire was used as auxiliary electrode, and the reference electrode was the silver/0.1 M silver nitrate couple (0.578 vs. NHE[40]).

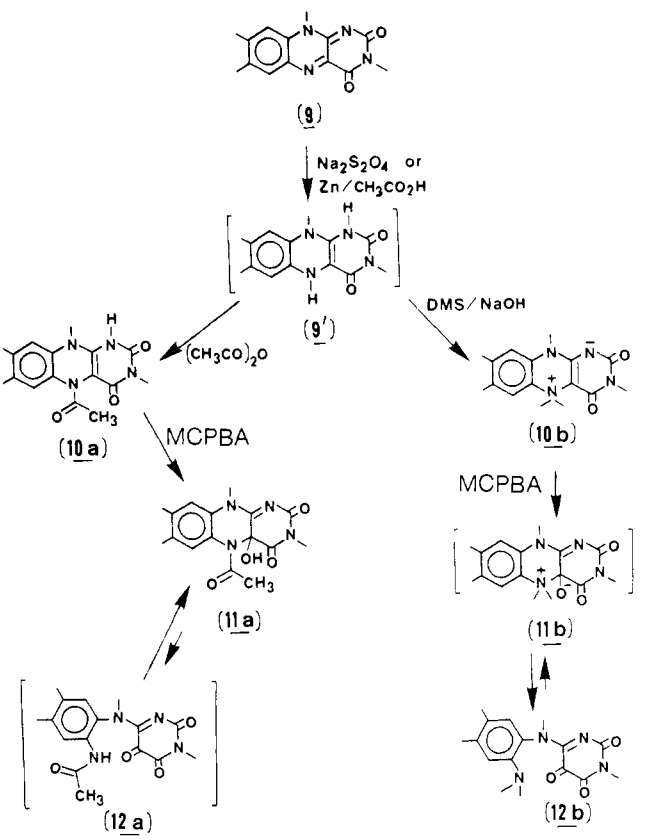
Calculations were done on a Hewlett-Packard 9825 A calculator. Programs used were developed in this laboratory by Drs. G. Eberlein, T. Eckert and P. Shannon.

Results

Our approach to the synthesis of 4a,5 ring opened flavins [6-(*N,N*-disubstituted amino)-5-oxo-3*H*,5*H*-uracils (**12**)] consisted of the complementary pathways of Schemes IV and V. The synthetic approach of Scheme IV was abandoned when it was found that neither **5a–c** nor **4a–c** could be converted to the desired 5-oxouracils (**12**). The approach in Scheme V consists of oxygenation of a N^5-substituted flavin (**10b**) to yield a 4a-substituted flavin (**11b**; not isolated) and ring opening. The 4a,5 ring opened flavin (**12b**) was obtained by this means (see Experimental Section).

The reduction of the 5-diazo-3*H*,5*H*-uracils (**4a–c**) to 5-oxo-3*H*,5*H*-uracil 5-hydrazones (**5a–c**) was accomplished by treatment

Scheme V



with either triphenylphosphine in dry aprotic solvent followed by acid hydrolysis or by prolonged stirring in triphenyl phosphite (Scheme IV). The use of the first method with α-diazo ketones has been reported previously.[41a] Reaction of the diazo compound with triphenylphosphine leads first to the triphenylphosphoranylidenehydrazine (cf. Scheme VI). In the case of **4b** the intermediate 3-methyl-6-(methylphenylamino)-5-(triphenylphosphoranylidenehydrazono)-3*H*,5*H*-uracil (**5b'**) was isolated. Because **5b'** is rather stable toward acid hydrolysis, the hydrazone **5b** is better prepared by stirring **4b** in triphenyl phosphite. Neither 6-(dimethylamino)-3-methyl-5-oxo-3*H*,5*H*-uracil 5-hydrazone (**5a**) nor its analogues 3-methyl-6-(methylphenylamino)-5-oxo-3*H*,5*H*-uracil 5-hydrazone (**5b**) and 6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil 5-hydrazone (**5c**)] could be hydrolyzed under mild or strongly acid conditions to give their corresponding 5-oxo-3*H*,5*H*-uracils (Scheme IV). Basic hydrolysis of **5a–c** was not considered due to the known susceptibility of the pyrimidine ring of alloxane-like derivatives to hydrolysis.[42,24a] α-Keto hydrazones have been successfully converted into α-diketones by reaction with nitrous acid.[41b] A one-pot synthesis of the desired product **12** (Scheme IV) was attempted by reaction of **4a** with triphenylphosphine followed by addition of $NaNO_2$ and acid. This procedure led to the isolation of the hydrazone **5a** even after prolonged reaction time, demonstrating the inertness of **5a** toward nitrous acid. This same procedure when applied to the phenyl-substituted diazo compound **4b** provided 3,10-dimethylisoalloxazine 5-oxide (**8a**) as the main product and a small yield of 3,10-dimethylisoalloxazine (**8b**) (Scheme VI). The latter most likely arises from the deoxygenation of **8a**.[33]

The stability of the 5-diazo group of the 5-diazo-3*H*,5*H*-uracils toward hydroxydediazoniations turned out to be the main obstacle

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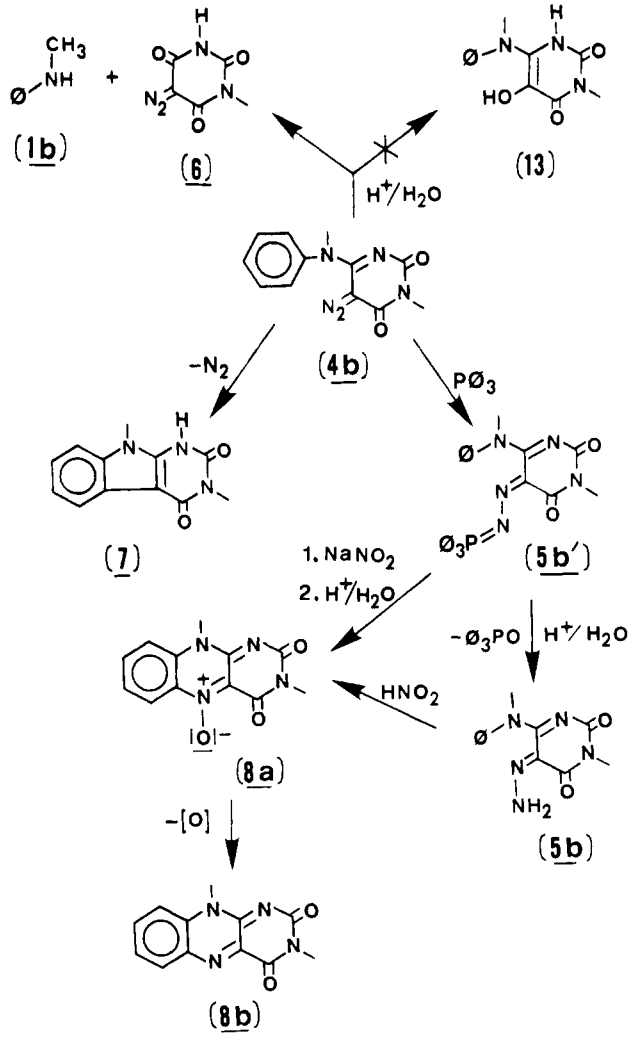
(40) Mann, C. K. "Electroanalytical Chemistry. A Series of Advances"; Bard, A.J., Ed.; Marcel Dekker: New York, 1969; Vol. III.

(41) (a) Bestmann, H.-J.; Buckschewski, H.; Leube, H. *Chem. Ber.* **1959**, *92*, 1345–1355. (b) Bestmann, H.-J.; Klein, O.; Gothlich, L.; Buckschewski, H. *Chem. Ber.* **1963**, *96*, 2259–2265.
(42) (a) Kwart, H.; Sarasohn, I. M. *J. Am. Chem. Soc.* **1961**, *83*, 909–919. (b) Kwart, H.; Spayd, R. W.; Collins, C. J. *J. Am. Chem. Soc.* **1961**, *83*, 2579–2580.

Scheme VI

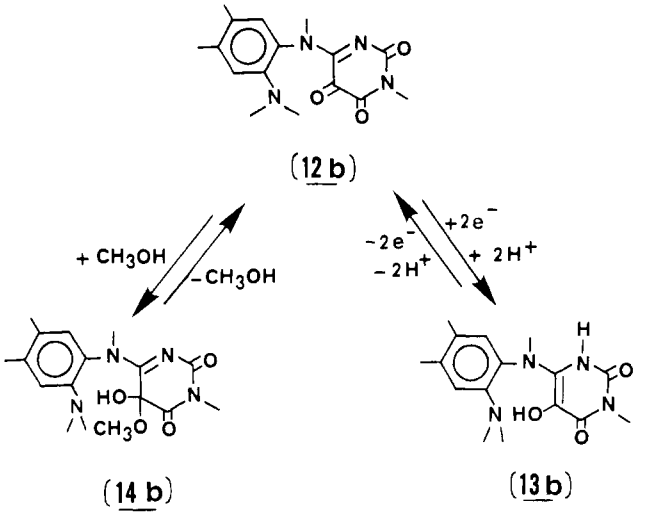


of the synthetic pathway of Scheme IV. Protonation of diazo compounds is known to be followed by dediazoniation and reaction with the solvent.[43] In the instance of **4b**, refluxing in 1.5 M H_2SO_4 for 70 h was required in order to completely decompose the diazo compound (followed by TLC) (Scheme VI). The isolated product (10% yield) proved to be 5-diazo-1-methylbarbituric acid (**6**) and not the 5-oxo-3*H*,5*H*-uracil resulting from autoxidation of the expected hydroxydediazonization product **13**. Thus, with **4b**, nucleophilic attack at C(6), followed by elimination of methylphenylamine is favored over the hydroxydediazoniation of position C(5). When **4b** was boiled in water containing Cu(I) catalyst,[44] the known[32] 3,9-dimethylpyrimido[4,5-*b*]indole-2,4-(1*H*,3*H*,9*H*)-dione (**7**) was obtained in 67% yield.

Dihydroflavins 10a and 10b Were Oxygenated by Reacting with *m*-Chloroperbenzoic Acid in Chloroform (see Scheme V; Experimental Section). With **10a**, an excess of per acid was required in order to get complete reaction of the starting material, while approximately equimolar amounts of **10b** and per acid were used since reactions of the desired product (**12b**) and **10b** with per acid compete. The reaction of **10b** was incomplete under these conditions. In both cases only moderate yields of the products (ca. 20% for **11a** and 30% for **12b**) could be obtained. Structural data (see Discussion) show that 5-acetyl-4a,5-dihydro-4a-hydroxy-3-methyllumiflavin (**11a**) had been obtained by oxygenation of **10a** but that in the case of **10b** a 4a,5 ring opening of the assumed intermediate (**11b**) to yield 6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (**12b**)

(43) Smith, A. B.; Dieter, R. K. *Tetrahedron* **1981**, *37*, 2407–2439.
(44) "Organikum"; VEB Deutscher Verlag der Wissenschaften: Berlin, 1972.

Scheme VII



had occurred. Formation of **12b** by 4a,5 ring opening is due, no doubt, to the improved leaving group character of the quaternary nitrogen at N(5).

Reactions of 6-[[2-(Dimethylamino)-4,5-dimethylphenyl]-methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (12b) Are Shown in Scheme VII. While stable in aprotic solvents like acetonitrile and chloroform, **12b** shows solvolysis in the protic solvents methanol and water. 6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-5-hydroxy-5-methoxy-3-methyl-3*H*,5*H*-uracil (**14b**), the methanol adduct of **12b**, was conveniently obtained by stirring **12b** in methanol and was isolated and purified by column chromatography over silica gel with aprotic eluents (chloroform/ethyl acetate) in 38% yield as the main product of methanolysis (for its structure, see Discussion). The isolation of *N*,*N*',*N*',4,5-pentamethyl-*o*-phenylenediamine (**1c**, see Scheme IV), as a byproduct, indicates that splitting of **14b** (or **12b**) occurs after addition of methanol to the C(6) position of **14b** (or **12b**). The diamine **1c** was also isolated as a main product of the hydrolysis of **12b**. Isolation of the hydrate of **12b**, which corresponds to the methanol adduct **14b**, was not attempted, nor was the fate of the pyrimidine ring of **12b** sought through studies of extensive methanolysis or hydrolysis (vide infra). The solvolytic lability of 5-carbonylpyrimidines, like alloxane, is well documented[42] (see Discussion). Attempts to derivatize the C(5)-carbonyl group of **12b** via formation of a hydrazone were unsuccessful. This failure is due to oxidation of hydrazine by **12b** which leads to the formation of the **13b** (vide infra). Thus, once formed, the hydrazone cannot be hydrolyzed to yield **12b** (see above) but **12b** cannot be employed in the synthesis of its hydrazone. A useful synthetic way to obtain 6-[[2-(dimethylamino)-4,5-dimethylphenyl]-methylamino]-5-hydroxy-3-methyluracil **13b** (reduced **12b**) is to shake the oxidized form **12b**, contained in a chloroform layer, intensively with aqueous dithionite solution and precipitate **13b** with diethyl ether after evaporation of the chloroform layer. The product was successfully recrystallized under an inert atmosphere from ethanol and converted again into starting material **12b** by autoxidation in aprotic solvents.

The solvolysis of 6-[[-2-(dimethylamino)-4,5-dimethylphenyl]-methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (12b) has been studied in protic solutions. **12b** is stable in the aprotic solvents chloroform and acetonitrile, which were used for obtaining structural data (see Discussion). Figure 1A shows the UV spectrum of **12b** in acetonitrile, displaying three maxima at 229, 245, and 342 nm. Remarkable is the absorption at longer wavelengths, which, although of low extinction, reaches beyond 500 nm. This long-wavelength absorbance is responsible for the orange-brown color of concentrated solutions of **12b**. The same spectral features are initially observed in methanolic and aqueous solution (Figure 1B,C), but further repetitive scans clearly indicate solvolytic reactions. The solvolysis of **12b** (followed at 342 nm) in spectral-grade methanol (Mallinckrodt) follows the first-order

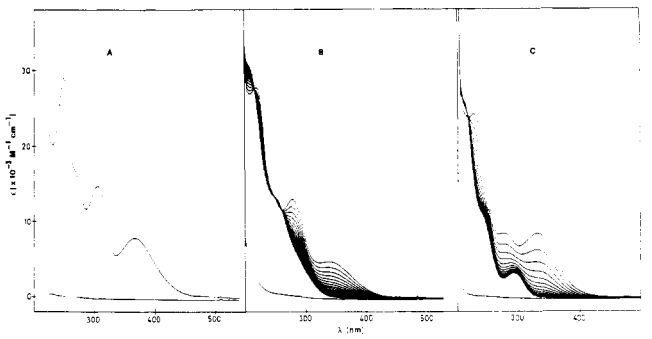


Figure 1. UV/vis spectra of **12b** (5×10^{-5} M) in acetonitrile (A), methanol (anhydrous, AR, 1% CH_3CN) (B), and phosphate buffer, pH 6.46 (0.02 M; μ = 1 with KCl, 1% CH_3CN) (C). The delay between repetitive scans is 988 s.

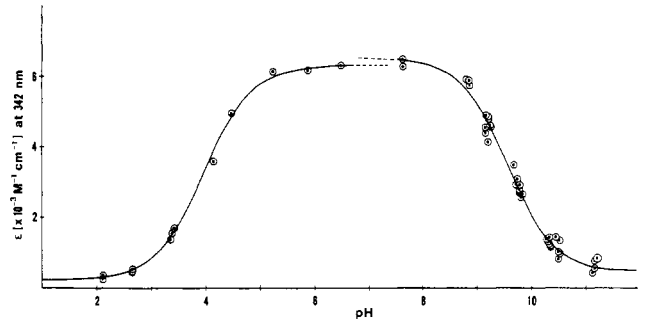


Figure 2. Extinction at 342 nm of 5×10^{-5} M solutions of **12b** in aqueous buffers (1% CH_3CN) vs. pH. The experimental points were obtained by extrapolation of the absorbance vs. time curves for hydrolysis to $t = 0$ (see Results). The solid lines are best fits, used for calculating pK_as (pK_a^1 = 3.98, pK_a^2 = 9.54).

rate law at all wavelengths (Figure 1B), and the pseudo-first-order rate constant (k_{obsd}) was determined to be 1.02×10^{-3} s^{-1} (30 ± 0.2 °C) in spectral-grade methanol. It was found that the rate of disappearance of **12b** is very dependent upon the source of methanol used. Rate changes by orders of magnitude indicate that formation of the adduct is catalyzed by acidic and basic impurities.

The Kinetics of the Hydrolysis of 12b Have Been Investigated as a Function of pH (H_2O Solvent; 1% in CH_3CN; μ = 1.00 with KCl; 30 ± 0.2 °C). In Figure 1C there are shown repetitive scans of the disappearance of **12b** at pH 6.46, where it has a stability maximum; the kinetics are pseudo first order when followed above 250 nm. The change of the position of the isosbestic point below 250 nm (not observed in methanolysis) indicates the reaction in H_2O to be more complicated than in methanol. Hydrolysis of **12b** was followed at 342 nm at pH values above 1.8 and at a wavelength of 270 nm below this pH. This requirement was dictated by the low extinction of **12b** in acid solution (see Figure 2).

Extrapolation of the absorbance vs. time traces for the disappearance of **12b** to $t = 0$, and plotting these initial absorbances vs. pH, results in the plot shown in Figure 2. The solid curves were obtained as best fits to the experimental points by using a computer program for pK_a determinations, which provided the values pK_{a_1} = 3.98 and pK_{a_2} = 9.54. These pK_a values (eq 7) are related to proton and HO^- addition to **12b**.

$$12bH^+ \underset{+H^+}{\overset{K_{a_1}}{\rightleftharpoons}} 12b \underset{+H^+}{\overset{K_{a_2}}{\rightleftharpoons}} 12bHO^- \qquad (7)$$

Values of the pseudo-first-order rate constants (k_{ly}) for hydrolysis of $\mathbf{12b_T}$ (=$\mathbf{12bH^+ + 12b + 12bHO^-}$) were obtained by extrapolation of pseudo-first-order rate constants (k_{obsd}) to zero buffer concentrations at constant pH. No buffer catalysis was detected below a pH of 6. Above this pH between a 10 and 20% change in k_{obsd} was detected for a change of total buffer concentration of 1 order of magnitude (0.2–0.02 M). The pH–log

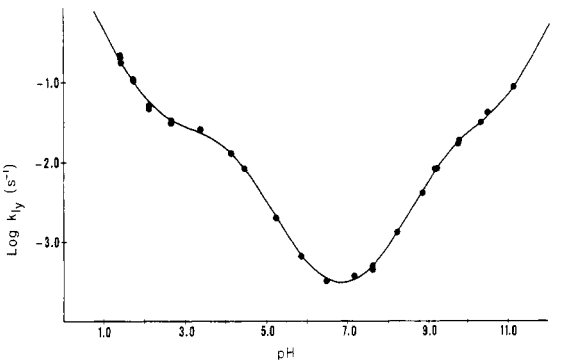


Figure 3. log k_{ly} vs. pH profile for hydrolysis of a 5×10^{-5} M solution of **12b** in aqueous solution (μ = 1 with KCl, 1% CH_3CN, 30 °C). The solid line is the best fit of eq 8 to the experimental points. For rate constants and pK_as, see Table I.

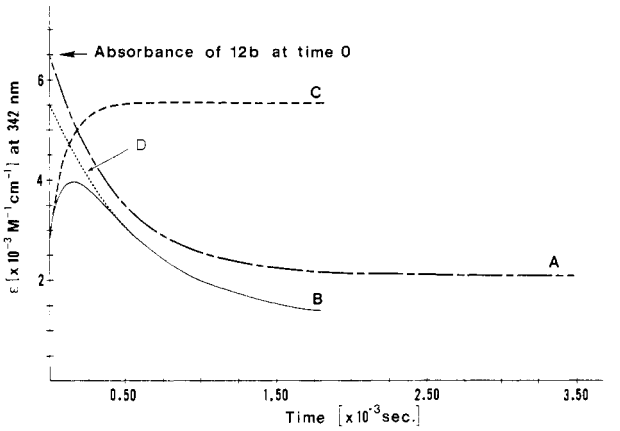


Figure 4. Line A represents the disappearance of **12b** (initially at 5×10^{-5} M) with time from a hydrazine hydrochloride buffer solution (0.02 M; pH 8.09, μ = 1 with KCl, 1% CH_3CN at 30 °C, anaerobic). The biphasic curve B represents the change of absorbance on admission of air to the spent reaction solution. Curves C and D are curve B separated into two first-order components.

Table I. Hydrolysis of 6-[[2-(Dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil (**12b**): Rate and Equilibrium Constants Obtained from Theoretical Line of Figure 3

$k_+^H = 4.17$ M^{-1} s^{-1}	pK_{a_1} = 4.13
$k^H = 3.43 \times 10^2$ M^{-1} s^{-1}	pK_{a_2} = 9.61
$k^{H_2O} = 2.09 \times 10^{-4}$ s^{-1}	
$k^{HO} = 4.64 \times 10^2$ M^{-1} s^{-1}	
$k_-^{HO} = 2.98 \times 10^1$ M^{-1} s^{-1}	

k_{ly} profile of Figure 3 is the best fit of the experimental points to the expression of eq 8

$$k_{ly} = k_+^H a_H^3 + k^H a_H^2 K_{a_1} + k^{H_2O} K_{a_1} a_H + k^{HO} K_w K_{a_1} + k_-^{HO} K_w K_{a_1} K_{a_2} / a_H / K_{a_1} K_{a_2} + K_{a_1} a_H + a_H^2 \qquad (8)$$

which was deduced from the rate law of eq 9

$$-\frac{d[\mathbf{12b_T}]}{dt} = k_+^H a_H[\mathbf{12bH^+}] + k^H a_H[\mathbf{12b}] + k^{H_2O}[\mathbf{12b}] + k^{OH}[HO^-][\mathbf{12b}] + k_-^{OH}[HO^-][\mathbf{12bHO^-}] \qquad (9)$$

by a material balance employing the definition of $\mathbf{12b_T}$. The rate constants obtained from the best fit are shown in Table I. Also obtained were the apparent equilibrium constants K_{a_1} and K_{a_2}, whose pK_{app} values of 4.13 and 9.61 compare well with the values previously obtained from a plot of initial absorbance vs. pH (3.98 and 9.54; Figure 2). A value of 1.419×10^{-14} was used for the autoprotolysis constant of water at ionic strength 1 and 30 °C (K_w).[38a]

Reduction of 12b by hydrazine (1% CH_3CN in H_2O; μ = 1 with KCl; 30 ± 0.2 °C) follows the first-order rate law (342 nm) when

$[NH_2NH_3^+ + NH_2NH_2] = [NH_2NH_{2_T}] \gg [\mathbf{12b}_T]$. Line A in Figure 4 shows the best first-order fit [$k_{obsd} = (2.29 \pm 0.03) \times 10^{-3}$ s^{-1}] of A_{342} vs. time when employing a solution 5×10^{-5} M in **12b** and 0.02 M in $NH_2NH_{2_T}$ at pH 8.09 under anaerobic conditions. Further, the absorbance (0.105) of the hydrazine-containing reaction solution after more than seven half-lives was found to be greater than the absorbance of the spent reaction solutions which did not contain hydrazine (0.019 at pH 8.22 and 0.008 at pH 7.62). This observation establishes that the product of hydrazine reaction is different from that of the hydrolysis reaction (see also Figure 7 under Discussion). In Figure 4 line B represents the best fit of a digital program for two consecutive first-order reactions (P → Q → R) to the experimental trace of A_{343} vs. time when the hydrazine reaction solution at t_∞ (line A) is subject to aeration. The first-order rate constants employed to generate line B are $k_1 = 9.55 \times 10^{-3}$, $k_2 = 1.67 \times 10^{-3}$. These kinetic results are in accord with the minimal reaction sequence of eq 10

$$\mathbf{12b} \xrightarrow{N_2H_4} \mathbf{13b}$$
$$\mathbf{13b} \xrightarrow{O_2} \mathbf{12b} + H_2O_2$$
$$\mathbf{12b} \xrightarrow{H_2O} \text{hydrolysis} \qquad (10)$$

where k_1 represents oxidation of **13b** and k_2 the competing reduction and hydrolysis of **12b** after admission of air. The individual reactions associated with k_1 and k_2 are represented by the lines C and D of Figure 4. Examination of the intercepts of the C and D lines on the absorbance axis reveals that 85% of **12b** has been reduced by hydrazine and is available for autoxidation, while 15% of **12b** has undergone hydrolysis. Further studies of the kinetics of reduction of **12b** by hydrazine were carried out (A_{342}) by employing five buffer dilutions (0.02–0.2 M) in hydrazine at the pH values 7.58, 7.76, 7.97, 8.29, and 8.97 ($\mu = 1.0$ with KCl). Solutions were prepared and pH determinations made in a N_2 atmosphere drybox containing the spectrophotometer. All reactions followed the first-order rate law. By extrapolation of A_{342} to $t = 0$, at any constant pH there was obtained an initial absorbance, which proved to be inversely proportional to the total concentration of hydrazine at any constant pH. This suggests the formation of an equilibrium between hydrazine, the 5-oxo-3*H*,5*H*-uracil **12b**, and a complex or compound formed by reaction of these components (eq 11). Further, the equilibrium is es-

$$\mathbf{12b}\cdot N_2H_4 \underset{}{\overset{K_D^{12b\cdot N_2H_4}}{\rightleftharpoons}} \mathbf{12b} + N_2H_4 \qquad (11)$$

tablished during the time of mixing. Plots of the pseudo-first-order rate constants for A_{342} disappearance at constant pH values in the vicinity of the pK_a of hydrazine (8.11)[41] vs. the total concentrations of hydrazine ($[N_2H_{4_T}]$) are shown in Figure 5. The linearity of the plots establish the reaction to be first order with respect to hydrazine at all pH values. The points at zero hydrazine concentrations are identical with the rate constants determined previously for the hydrolysis of **12b**. These results are explainable through either of the kinetically identical mechanisms of eq 12a and 12b. The calculation of $K_D^{12bN_2H_4}$ as a function of pH is

$$\mathbf{12b} + N_2H_4 \underset{K_D^{12b\cdot N_2H_4}}{\rightleftharpoons} \mathbf{12b}\cdot N_2H_4$$
$$\downarrow k_r$$
$$\mathbf{13b} \qquad (12a)$$
$$-\frac{d[\mathbf{12b}]}{dt} = \frac{k_r[N_2H_4][T]}{[N_2H_4] + K_D^{12b\cdot N_2H_4}}$$

$$\mathbf{12b} + N_2H_4 \underset{K_D^{12b\cdot N_2H_4}}{\rightleftharpoons} \mathbf{12b}\cdot N_2H_4$$
$$\downarrow k_2$$
$$\mathbf{13b} \qquad (12b)$$
$$-\frac{d[\mathbf{12b}]}{dt} = \frac{k_2 K_D^{12b\cdot N_2H_4}[N_2H_4][T]}{[N_2H_4] + K_D^{12b\cdot N_2H_4}}$$

$$[T] = [\mathbf{12b}] + [\mathbf{12b}\cdot N_2H_4]$$

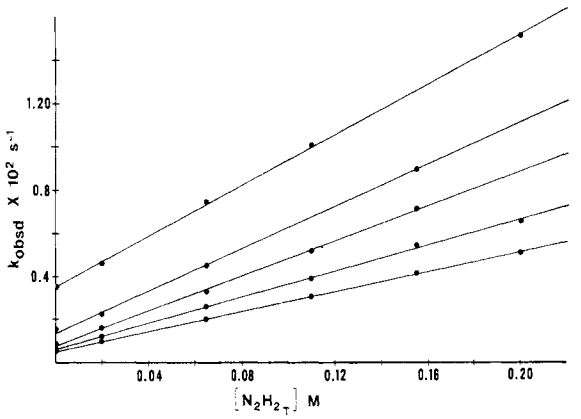


Figure 5. Dependence of the observed pseudo-first-order rate constants for disappearance of **12b** (initial concentration of 5×10^{-5} M, 1% CH_3CN; $\mu = 1$ with KCl, 30 °C) in anaerobic hydrazine hydrochloride buffers on the total concentration of hydrazine at pH values of 7.58, 7.76, 8.29, and 8.73.

beclouded by the equilibrium formation of $\mathbf{12b}HO^-$ (eq 7). The observation of a first-order dependence in the rate of **12b** reduction by hydrazine requires $K_D^{12bN_2H_4} > 1$. The plots of Figure 5 find explanation through the rate equation 13. The slopes of the linear

$$k_{obsd} = k_{ly} + k_r \frac{K_a^{N_2H_4}}{K_a^{N_2H_4} + a_H}[N_2H_{4_T}] \qquad (13)$$

plots of Figure 5 are pH-dependent apparent second-order rate constants which when divided by the mole fraction of hydrazine as the free base at constant acidity [i.e., $K_a^{N_2H_4}/(K_a^{N_2H_4} + a_H)$] equal the true second-order rate constant. Values of k_r (eq 12a) = $k_2 K_D^{12bN_2H_4}$ (eq 12b) vary between 8×10^{-2} and 12×10^{-2} M^{-1} s^{-1}.

Oxidation of 6-[[2-(dimethylamino)-4,5-dimethylphenyl]-methylamino]-5-hydroxy-3-methyluracil (13b) (4.07×10^{-5} M) in air-saturated acetonitrile is accompanied by the disappearance of absorbance attributed to **13b** (λ_{max} at 307 nm) and the appearance of **12b** (λ_{max} at 342, 277, and 220 nm). The three isosbestic points (330, 290, 210 nm) exclude the buildup of an intermediate. The conversion of **13b** into **12b** followed pseudo-first-order kinetics giving a k_{obsd} of $(1.53 \pm 0.07) \times 10^{-3}$ s^{-1}. The compound **13b** undergoes autoxidation in aqueous solution. A repetitive scan of the O_2 oxidation of **12b** at a pH of 2.64 in water is characterized by the disappearance of the 307-nm absorbances of **13b** and a plot of absorbance vs. time provides a sigmoid curve indicative of an autoxidative process.

The electrochemical investigation of the redox properties of **12b** and **13b** was conducted in acetonitrile due to the instability of **12b** in protic solvents and the insolubility of **13b** in neutral buffers. Tetraethylammonium perchlorate (0.1 M) was used as the supporting electrolyte, and the potentials were measured *vs.* the Ag/0.1 M $AgNO_3$ electrode. The values are given vs. the normal hydrogen electrode (NHE). A potential of 0.578 V was assumed for the reference electrode used (see Experimental Section). By using thin-layer cyclic voltammetry[39] it was possible to calculate the number of electrons transferred from the areas of the peaks, thereby distinguishing between one- and two-electron transfer steps. The reduction of a 4.74×10^{-4} M solution of **12b** showed a quasi-reversible one-electron reduction wave at −0.48 V when scanned at 1 mV/s (Figure 6). The corresponding oxidation wave had a peak potential of −0.36 V. The difference between the potentials (120 mV) shows the quasi-reversibility of this first one-electron transfer, as a difference of only 30 mV is expected for the oxidation and reduction wave corresponding to a reversible one-electron transfer. When the scan speed is raised, the oxidation and reduction peak potentials move apart, again confirming the assumption of a quasi-reversible one-electron transfer. A second electron transfer was seen on scanning further to more negative potentials. This amounted to a broad reduction wave between −0.96 and −1.51 V. The corresponding oxidation wave was ob-

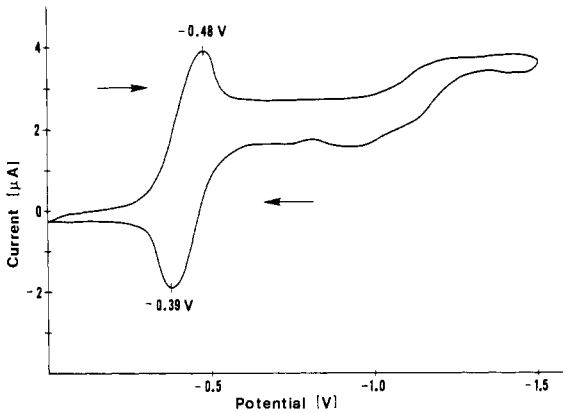
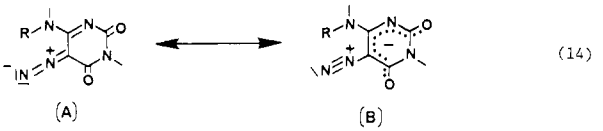


Figure 6. Thin-layer cyclic voltammogram (scan rate 1 mV/s) for the reduction of a 4.74×10^{-5} M solution of **12b** in acetonitrile–0.1 M tetraethylammonium perchlorate. Potentials are given vs. the normal hydrogen electrode (NHE).

served between −1.28 and −0.80 V. The integral of the area showed the transfer of one electron (ca. 0.9). The calculated number of electrons transferred in the peak of more negative potential diminished with increasing scan speed. When a solution containing 4.07×10^{-4} M **13b** was scanned from a potential of −1.3 V toward more anodic potential values, only one irreversible oxidation wave, at −0.58 V, could be observed at a scan speed of 1 mV/s, its area accounting for two electrons transferred. Shape and position of this irreversible two electron transfer wave were strongly dependent on the scan speed. When the same solution was scanned by using thick layer (i.e., conventional) cyclic voltammetry from cathodic to anodic potentials, the first oxidation wave was observed at −0.50 V when a scan speed of 10 mV/s was used. Further irreversible oxidation peaks were observed at +0.50 and +1.18 V. The first (irreversible as it showed no corresponding reduction wave) oxidation peak (at −0.50 V with a scan speed of 10 mV/s) moved to more positive potentials when the scan speed was raised, giving a peak position of −0.27 V at a scan speed of 100 mV/s. In summary, in the aprotic solvent acetonitrile the first one-electron reduction–oxidation of **12b** (4.74×10^{-4} M) is quasi-reversible ($E_{m_1} = 0.42$ V). Transfer of the second electron occurs in a process which appears chemically reversible but electrochemically nonreversible with a very approximate E_{m_2} of −1.1 V. Anodic sweeps using the reduced form of **12b** (i.e., **13b**) at 4.07×10^{-4} M showed an irreversible two-electron oxidation wave.

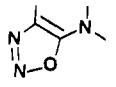
Discussion

5-Diazo-3*H*,5*H*-uracils belong to the general class of α-diazo carbonyl compounds, which are more stable than their aliphatic counterparts due to resonance between the diazo carbonyl form (eq 14, A) and the diazonium enolate (eq 14, B).[45] The stability



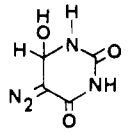
(A) (B) (14)

of diazouracils, diazobarbituric acids, and other cyclic diazoketones has led to the proposal of valence isomeric pyrimido[5,4-*d*]-1,2,3-oxadiazolo structures,[46,45b] (structure V in the case of the



V

compounds **4a–c**). In general, however, the existence of such annelated 1,2,3-oxadiazoles (V) has been excluded by the observation of strong IR bands in the 2000–2300-cm^{-1} region, which can be ascribed to a diazo group but not to a 1,2,3-oxadiazole.[47,46b-i] The same argument can be used to exclude structure V in the case of **4a–c** [strong IR bands at 2130 (**4a**), 2140 (**4b**), and 2130 (**4c**) cm^{-1}]. The diazonium enolate structure (B) might be expected to predominate over (A) in the case of the 5-diazo-3*H*,5*H*-uracils **4a–c**, because in this form the negative charge can be delocalized over the pyrimidine ring system. This does not, however, appear to be the case. The wavenumber of the stretching vibration of the diazo/diazonium group has been correlated to the relative importance of the contributions of the carbonyl diazo (e.g. A) vs. the enolate diazonium (e.g., B, eq 14) structure. According to Fahr's compilation[47b] absorptions at 2300–2240 cm^{-1} are observed for aromatic diazonium salts, at 2190–2150 cm^{-1} for cyclic carbonyl diazo compounds, at 2160–2140 cm^{-1} for acyclic α,α′-dicarbonyl diazo compounds, at 2130–2110 cm^{-1} for simple α-diazo carbonyl compounds, and finally at 2105–2010 cm^{-1} for aliphatic diazo compounds. The IR bands of the compounds **4a–c** would therefore indicate that the contribution of the diazonium resonance structure B is not much more significant than in simple α-diazo carbonyl compounds (despite the possibility of the extensive delocalization of the negative charge on the pyrimidine ring). This is in contrast to unsubstituted 5-diazouracil, whose absorbance at 2190 cm^{-1} has been ascribed to a predominance of the 5-diazonium uracilate structure (corresponding to B) over the 5-diazo-5*H*-uracil structure (corresponding to A).[47b] Unfortunately, this comparison is hampered, in the case of unsubstituted uracil, by the uncertainty of the position of the proton [N(1) or N(3)]. Previous investigations[47b] have favored protonation of position N(1) with the C(5,6) double bond fixed. This preference is supported by the difference in the *NN* vibrations of unsubstituted 5-diazouracil and those of the compounds **4a–c**, because the (3*H*,5*H*)-uracil structure is fixed in **4a–c** by the methyl group on N(3). That hydration is the cause of the low values of the NN vibration of the compounds **4a–c** [cf. a value of 2150 cm^{-1} for hydrated 5-diazouracil (VI)] is excluded by the fact that water did not show up in the elemental analysis (see Experimental Section).



VI

The mechanism of the acid hydrolysis of α-carbonyl diazo compounds and other related species has been well studied.[43,48] It has been concluded that protonation of the carbon to which the diazo group is bound leads to facile hydroxydediazoniation while protonation of a vinylogous position leads to stable diazonium salts.[48,49] The ease of hydroxydediazoniation has been correlated to the NN vibration band,[47b] as the predominant position of protonation was thought to be influenced by the relative importance of the resonance contributions by the diazocarbonyl (A)

(45) (a) Patai, S., Ed. "The Chemistry of Diazonium and Diazo Groups", Wiley: New York, 1978. (b) For a review of heterocyclic diazo compounds, see: Elnagdi, M. H.; Zayed, E. M.; Abdou, S. *Heterocycles* **1982**, *19*, 559–578.

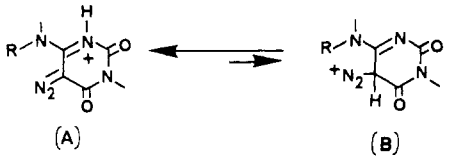
(46) (a) Johnson, T.-B.; Baudisch, O.; Hoffmann, A. *Ber. Dtsch. Chem. Ges. A* **1931**, *64*, 2629–2631. For a discussion of structures and reactions (in alkaline solutions) of 5-diazouracils, see: (b) Hügi, R.; Pfleiderer, W. *Justus Liebigs Ann. Chem.* **1972**, *359*, 76–83. (c) Thurber, T. C.; Townsend, L. B. *J. Heterocycl. Chem.* **1972**, *9*, 629–636. (d) Thurber, T. C.; Townsend, L. B. *J. Am. Chem. Soc.* **1973**, *95*, 3081–3082. (e) Thurber, T. C.; Pugmire, R. J.; Townsend, L. B. *J. Heterocycl. Chem.* **1974**, *11*, 645–647. (f) Thurber, T. C.; Townsend, L. B. *J. Org. Chem.* **1976**, *41*, 1041–1051. (g) Romani, S.; Klötzer, W. *J. Heterocycl. Chem.* **1978**, *15*, 1349–1350. (h) Romani, S.; Schönholzer, P.; Klotzer, W. *J. Heterocycl. Chem.* **1979**, *16*, 293–295. (i) Romani, S.; Virleitner, G.; Klötzer, W. *Liebigs Ann. Chem.* **1979**, 1518–1522. (j) Klötzer, W.; Romani, S. *Liebigs Ann. Chem.* **1981**, 1429–1432, and references cited therein.

(47) (a) Fahr, E. *Justus Liebigs Ann. Chem.* **1958**, *617*, 11–19. (b) Fahr, E. *Justus Liebigs Ann. Chem.* **1960**, *638*, 1–32.

(48) More O'Ferrall, R. A. *Adv. Phys. Org. Chem.* **1967**, *5*, 331–399.

(49) (a) Reimlinger, H. *Angew. Chem., Int. Ed. Engl.* **1963**, *2*, 482–483. (b) Reimlinger, H. *Chem. Ber.* **1966**, *97*, 3493–3502.

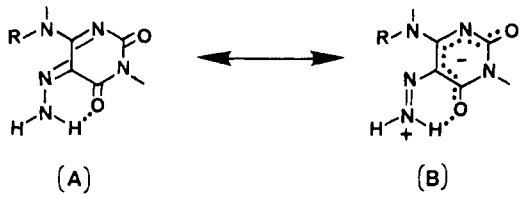
and of the diazonium enolate structure (B, eq 14). Thus, diazoalkanes (2105–2010 cm^{-1}) react readily with weak acids, α-diazo ketones (2130–2110 cm^{-1}) are stable in acetic acid but react with dilute mineral acids, and cyclic diazo ketones (2190–2150 cm^{-1}) are even stable in concentrated hydrochloric acid at low temperature (unfortunately no further experimental details are given for the latter statement). The compounds **4a–c**, which show bands between 2130 and 2140 cm^{-1}, obviously do not fit well into this correlation, as their stability toward 1–2 M mineral acids even at reflux temperatures (see Results) places them with the cyclic diazo ketones and not with the simple α-diazo ketones. By analogy with flavins,[50] the predominant position of protonation can be assumed to be N^1, and the N^1-protonated species (A) seems not to be in equilibrium with appreciable amounts of a C^5-protonated form (B; eq 15). If the position of the NN band correlates well



(15)

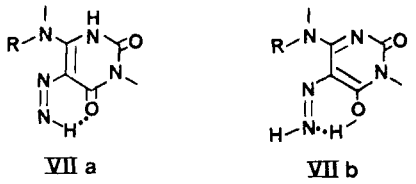
with the relative importance of the resonance structures A and B in eq 14, those relative contributions to the unprotonated form seem not to predict well the relative weight of the predominant positions of protonation.

5-Oxo-3*H*,5*H*-uracil 5-hydrazones are derivatives of the desired models for 4a,5 ring opened flavin. Their structures are represented by the two resonance forms A and B of eq 16. As al-



(16)

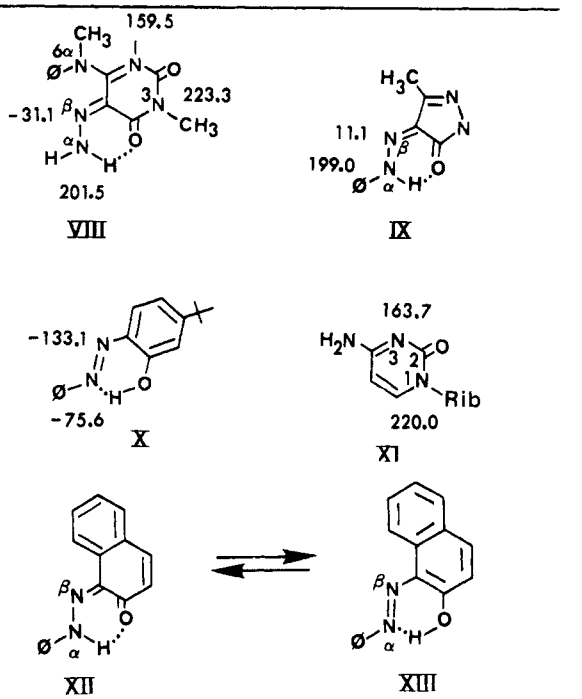
ternatives, the tautomeric diazene structures VII have to be



considered, or an equilibrium between the hydrazone represented by the resonance forms A and B (eq 16) and its diazene tautomers VII. A careful assignment of the structures is of some interest because A and B of eq 16 are isoelectronic and isosteric with corresponding resonance forms of the carbonyl oxide (cf. formula I in Scheme I) postulated by Hamilton as the active "oxene" carrier in his proposal of the mechanism for the flavin and pterin monooxygenase enzymes.

The ^{15}N NMR spectra of **5b** [3-methyl-6-(methylphenylamino)-5-oxo-3*H*,5*H*-uracil 5-hydrazone] were obtained in order to provide assured structural assignments and are presented (together with signals of reference compounds) in Table II. Four resonances were observed in the proton-coupled and decoupled ^{15}N spectra at −31.1, 159.5, 201.5, and 223.3 ppm. The resonance at 201.5 ppm was split into a doublet of doublets ($^1J_{H-^{15}N}$ = 94 and 92 Hz or 104 and 92 Hz) in the coupled spectrum. The only nitrogen which would have this chemical shift and coupling pattern is N^α of the hydrazone VIII (cf. hydrazone IX; only one resonance form shown); N^α in either diazene, VIIa, or VIIb would have a shift downfield from the standard of HNO_3 and appear only as a doublet (cf. diazene X). The chemical shifts of the other resonances are also consistent for the hydrazone structure VIII and

Table II. ^{15}N NMR Shifts of 5b and Related Compounds



TEMP	N_α	N_β	Δ	% HYDR
330°K	101.8	-38.9	140.7	65%
290°K	120.0	-29.0	149.0	72%
230°K	152.0	-10.8	162.8	83%

the assignments (gained by comparison with the models IX, X,[51a] and XI[51b]) are shown in formula VIII. $N^{6\alpha}$ is a tertiary arylamine, which should have a long relaxation time and hence was not observed under the experimental conditions. The coupling pattern and chemical shifts clearly rule out the diazene structure VIIa. However, a tautomeric equilibrium could possibly exist between the hydrazone VIII and the diazene VIIb to give rise to the observed shifts. The tautomeric equilibrium of similar compounds, XII ⇌ XIII, has been previously measured by ^{15}N NMR.[51a] A model hydrazone, IX, and a model diazene, X, were used[51a] to determine the limiting shifts. Although in the model hydrazone, IX, N^α has a phenyl substituent which will shift the resonance to lower field than N^α in VIII, it is evident from the high-field position of the N^α resonance and the difference (Δ) between the N^α and N^β resonance that the hydrazone predominates over the diazene (see Table II). This is in accordance with the assumption that diazene structures are reactive intermediates in the hydrodediazonization of uracils (see, for example, ref 46j). We did not observe any hydrodediazonizations. Of some interest is the high-field value observed for N^β of **5b** (see formula VIII) when compared to N^β of the hydrazone model IX. We think that this indicates an important contribution of the resonance structure B to the resonance as described by eq 16. A predominance of resonance structure B (eq 16) could explain a lack of nucleophilicity of the nitrogen N^α of our compounds, manifested in the poor reactivity of **5a** toward nitrous acid, which prevented its conversion into a 5-oxo-3*H*,5*H*-uracil.

The Synthesis of a 5-Oxo-3*H*,5*H*-uracil Was Realized As Shown in Scheme V. In regard to the reactions of Scheme I, the hypothetical 4a,5 ring opened flavin (II) can be regarded to be in equilibrium with a 4a,5-hydrate (pseudobase) of oxidized flavin (5-HFl-4a-OH). Flavin 4a-pseudobases are readily obtained by

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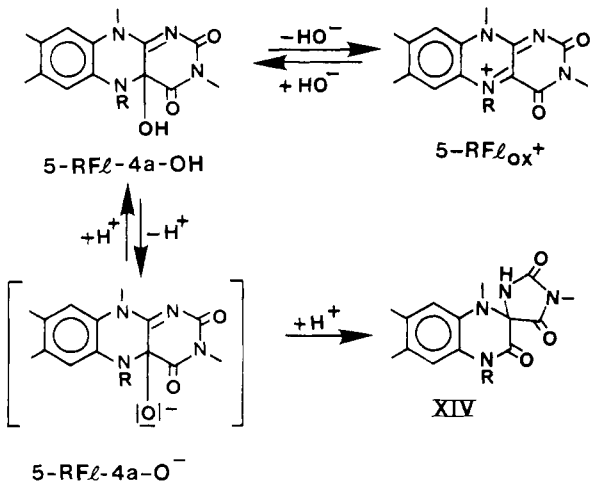
(51) (a) Lycka, A.; Snobl, D.; Machacek, V.; Veccru, M. *Org. Magn. Reson.* **1981**, *16*, 17–19. (b) *Annu. Rep. NMR Spectrosc.* **1981**, *11b*, 1–502.

Scheme VIII



HO^- addition to N^5-monoalkylated flavins ($HO^- + 5\text{-}RFl_{ox}^+ \rightleftharpoons$ 5-RFl-4a-OH, Scheme VIII) and are well characterized. The predominant reactions of the N^5-alkyl 4a-pseudobases are reversal of their formation by loss of the HO^- moiety[13b,35,52a] and 4a,10a ring contraction in the base to yield 10a-spirohydantoin (XIV).[52b] In order to avoid spirohydantoin formation by cleavage of the C^4–C^{4a} bond and to obtain C^{4a}–N^5 bond cleavage, we have resorted to the quaternization of the N^5-nitrogen of the flavin. By doing so the C^{4a}–N^5 cleavage of the tetrahedral 4a-pseudobase is favored since it involves elimination of a neutral amino function in concert with carbonyl group formation. The conversion of **10b** into a flavin 4a-pseudobase (Scheme V) was accomplished by hydroxylation with *m*-chloroperbenzoic acid. The (zwitterionic) intermediate **11b** could not be isolated or detected, and **12b** was obtained as the immediate end product. The formation of **12b** marks the first instance of 4a,5 ring opening of a flavin and the first synthesis of a N,N-disubstituted 6-amino-5-oxo-3*H*,5*H*-uracil. **12b** differs from the enzyme-bound intermediate postulated by Hamilton[18] and Massey et al.[21] for flavomonooxygenases and Kishore and Snell[4] for a flavodioxygenase (II, Scheme I) only by the replacement of the three hydrogens in positions N(3) and N(5) and the ribityl side chain on N(10) by methyl groups. Attempts to open the C^{4a}–N^5 bond of 3-methyllumiflavin by N^5-acetylation followed by treatment with *m*-chloroperbenzoic acid provided the 4a-pseudobase **11a** (Scheme V).

The reactions of the 5-oxo-3*H*,5*H*-uracil 12b, as outlined in Scheme VII, are those anticipated for such a compound based upon the known chemistry of alloxane (cf. Scheme III). In contrast to alloxane, where only the covalent hydrate form can be obtained conveniently, and the dehydrated form has to be made by sublimation and careful exclusion of water,[53] a solution of **12b** in chloroform can be washed with water without concern for its covalent hydration. Treatment of **12b** with methanol leads to the adduct **14b**, which is formulated as a C^5 adduct similar to hydrates of alloxane.[42] This structure is furthermore supported by the mass spectrum of **14b** (see Experimental Section), which shows (immediately after the molecular ion) the loss of a fragment corresponding to $C^5(OH)OCH_3$, which can be easily explained by a C^5 adduct, but is not to be expected from a C^6 adduct, which is usually found with substituted uracils (cf. VI).[46b-j] With time, further methanolysis occurs, leading to the production of the phenylenediamine **1c** and products resulting from solvolysis of the pyrimidine ring. The compound **1c** is also a product of hydrolysis in water. Reduction of **12b** with dithionite in the biphasic system chloroform/water has led to the isolation of the dihydro form of **12b**, 6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-5-hydroxy-3-methyluracil (**13b**) (Scheme VII).

The structure of 6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-3-methyl-5-oxo-3*H*,5*H*-uracil as given in formula **12b** of Schemes V and VII is substantiated in aprotic solvents by IR, ^1H NMR, and ^{13}C NMR spectra and elemental analysis. The IR spectrum shows three bands at 1720, 1700, and 1665 cm^{-1} (cf. Experimental Section), which are consistent with three C=O stretching vibrations. By comparison to the C=O stretching bands observed for the C^2- and C^4-carbonyl groups of flavins,[54] the absorptions at 1700 and 1665 cm^{-1} are assigned respectively to the C^4 and C^2 stretching vibrations, which leaves the band at 1720 cm^{-1} for the C^5-carbonyl group. All protons are accounted for in the ^1H NMR with their correct integrals and can be compared to their equivalents in the 5-nitrouracil **3c**, the 5-diazo-3*H*,5*H*-uracil **4c**, and the hydrazone **5c**. The methyl groups at N^3 and C^6–N are assigned by comparison with the corresponding data of flavin derivatives.[55] The δ value of 2.51 (singlet, six protons) for the dimethylamino group at position C(2′) clearly shows it to be comparable to the corresponding one in *N*,*N*′,*N*′,4,5-pentamethyl-*o*-phenylenediamine (**1c**, δ = 2.62), excluding the betaine **11b** (Scheme V) as a possible alternative. The methyl groups bound to the quaternary nitrogen of the hypothetical **11b** should give a ^1H NMR signal similar to those of the quaternary nitrogen N(5) of the 1,5-dihydro-3,5,5-trimethyllumiflavin (**10b**, Scheme V) which shows a signal at δ = 4.12.[35] Direct evidence for the hydration status of the C^5-carbonyl in the solvent chloroform has been gathered from the ^{13}C NMR.

The position of the ^{13}C NMR signals of four compounds (**12b**, **11a**, **10b**, and **5a**) obtained during this work (see Experimental Section) can be compared to published data of 3-methylriboflavin and of a N^5-blocked 4a-pseudobase of lumiflavin.[11b] Six signals of compound **12b** fall into the region between 120 and 140 ppm and can be conveniently assigned to the six carbons of the aromatic ring. By the same analogy, the carbons of a pyrimidine ring are expected to fall between 145 and 165 ppm. Four signals corresponding to the carbons C^2, C^4, C^5, and C^6 of **12b** are found between 150 and 165 ppm. No signal is found in the region between 50 and 115 ppm, where a signal caused by an sp^3-hybridized C^{4a} would be expected from the data of known flavin 4a adducts[11b] and 4a-substituted 5-deazapterines.[17a,b] The carbonyl group at C^5 is therefore firmly established. This excludes a covalent hydrate (as observed for alloxane) or a zwitterionic intramolecular adduct as **11b** (cf. Scheme V). The only signals for **12b** corresponding to sp^3-hybridized carbons can be assigned to the two methyl groups bound to the aromatic ring, to the two methyl groups bound to N^3 and C^6–N, and to the dimethylamino group, whose signal at 43.0 ppm excludes a quaternary nitrogen, which would be formed if **12b** existed as the zwitterionic pseudobase **11b**. The presence of a quaternary nitrogen would show itself by a signal at higher field as it is observed at 58.0 ppm for the quaternary nitrogen of **10b**. Another interesting feature of the ^{13}C NMR spectrum of **10b** is the signal at 97.1 ppm. As there are only three signals, which can be assigned to carbons in the pyrimidine ring, this signal must represent the C^{4a}-carbon, its upfield position caused by its high electron density. A signal at ~105 ppm is observed for the 4a-carbon of dihydroflavins.[11b] High electron density at C^5 (which corresponds to C^{4a} in flavins) is also expected for the hydrazones **5a–c** (see discussion of the ^{15}N NMR data), due to contributions of resonance structure B (eq 16). This is well reflected in the upfield position of one signal (117.0 ppm) of compound **5a** which was therefore assigned to C^5. In conclusion it can be said that the 5-oxo-3*H*,5*H*-uracil structure of compound **12b** is firmly established in aprotic media. The structural data of the N^5-blocked **11a** are consistent with its formulation as a flavin derivative (see formula **11a** in Scheme V), but as it analyzes as a hydrate, it would also be consistent with its formulation as a ring-opened hydrate, but we do not think that a change in the substitution of $C^{2'}$–N of a ring-opened form would

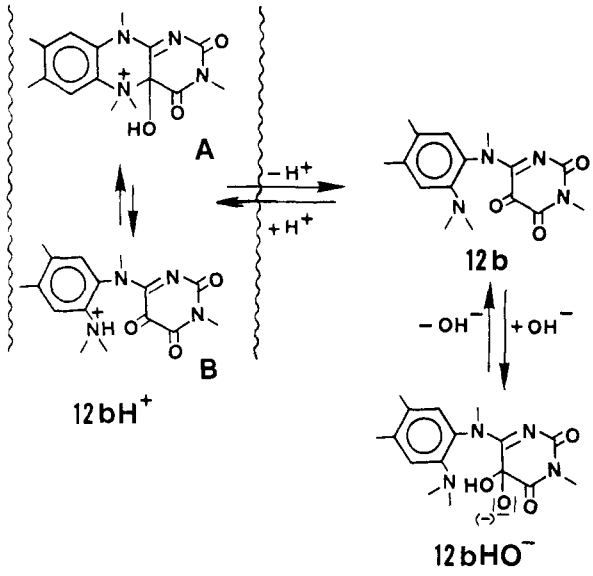
(52) (a) Kemal, C.; Bruice, T. C. *J. Am. Chem. Soc.* **1976**, *98*, 3955–3964. (b) Iwata, M.; Bruice, T. C.; Carrell, H. L.; Glusker, J. P. *J. Am. Chem. Soc.* **1980**, *102*, 5036–5099.

(53) Clark-Lewis, J. W.; Moody, K. *Aust. J. Chem.* **1970**, *23*, 323–328.

(54) Dudley, K. H.; Ehrenberg, A.; Hemmerich, P.; Muller, F. *Helv. Chim. Acta* **1964**, *47*, 1354–1383.

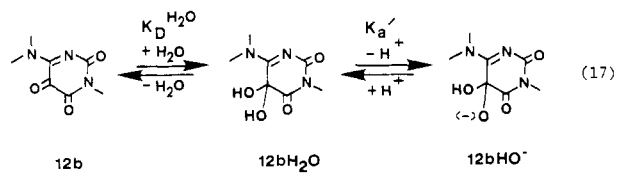
(55) Grande, H. J.; van Schagen, C. G.; Jarbandhan, T.; Muller, F. *Helv. Chim. Acta* **1977**, *60*, 348–366.

Scheme IX



drastically alter the hydration equilibrium of C^5=O. Pending further investigation, we therefore prefer to formulate **11a** as a flavin.

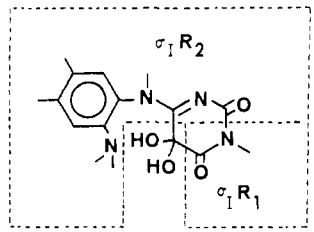
The structures and equilibria of 12b in aqueous solution can be deduced from the kinetics of its hydrolysis (see Results) and are shown in Scheme IX. The initial UV/vis spectrum of the repetitive scan in Figure 1c at pH 6.46 is similar enough to the corresponding one in acetonitrile to warrant the conclusion of the predominance of structure B over the zwitterionic flavin formula (**11b** in Scheme V) and the neutral C_5-hydrate in the neutral pH range. The value of pK_a^1 in the acid range (3.98 from the initial absorbance at 342 nm, Figure 2; 4.13 as the apparent pK_a in the pH–log k_{ly} profile for hydrolysis, Figure 3) can be assigned to the protonation of the dimethylamino group at $C^{2'}$ leading to species **B**. The value of pK_a^1 corresponds well to that of *N*,*N*-dimethylaniline (5.15)[37] when taking into account the electron-withdrawing nature of the pyrimidine ring at the neighboring $C^{1'}$–N. On the other hand, the big change in absorbance at 342 nm accompanying protonation (Figure 2) may well signal the formation of the cationic flavin pseudobase A (Scheme IX). The formation of a hydrate of form B is another possibility. The decrease in the initial absorbance (Figure 2) with increase in pH at high basicity (pK_a^2 = 9.54 from initial absorbance at 342 nm; 9.61 as apparent pK_a from the pH–log k_{ly} profile for hydrolysis, Figure 3), cannot be due to proton dissociation and must, therefore, be due to addition of HO^- to the C^5 position. Thus, pK_a^2 results from the readily reversible addition of hydroxide ion to the 5-oxo-3*H*,5*H*-uracil **12b** to form **12b**·H_2O and the ionization of the hydrate **12b**H_2O to **12b**HO^- (eq 17). In eq 17 the value of $K_D^{H_2O}$



(17)

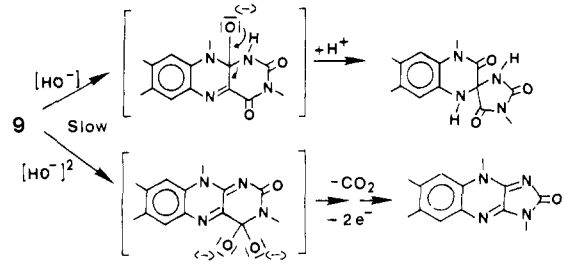
$$pK_a^2 = -\left[\log\left(1/K_D^{H_2O}K_a'\right)\right]$$

is not likely to deviate greatly from 10^{-1} so that pK_a' is ~8.5. A value of ~8.5 for pK_a' is most reasonable as can be deduced from the free energy relationship (pK_a = 14.19–8.2σ_I) found for the pK_a's of gem diols.[56] The ρ_I of –8.2 is obtained from the original –1.315 by correction with the factor 6.23 and allows the use of the σ_I values tabulated by Charton.[57] The substituents R_1 and R_2 in $R_1R_2C(OH)_2$ are expected to decrease the pK_a of one of the hydroxyl groups to 8.5 if the sum of the σ_I values for R_1 and R_2 in **12b**·H_2O equals 0.58. The σ_I value for the substituent



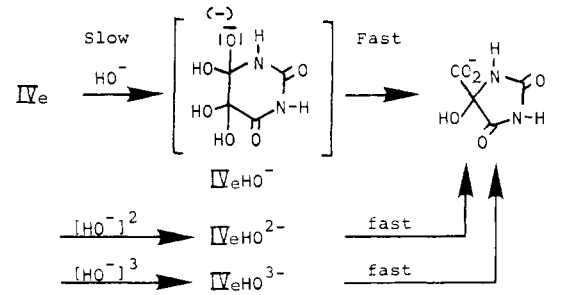
H_2N–CO– is given by Charton as +0.27 so that the σ_I for R_2 need only be ~0.3 in order that the pK_a of the gem diol functionality be equal to 8.5. A σ_I of ~0.3 for R_2 of **12b**·H_2O is most reasonable. In conclusion, both spectral titration of **12b** and the kinetics for its hydrolysis in aqueous solution necessitate the assumption of the rapid acid–base equilibria associated with pK_a^1 and pK_a^2 (prior to hydrolysis) as depicted in Scheme IX.

The kinetics of the hydrolysis of 12b may be compared to that of 3-methyllumiflavin[58] and 3-methylalloxane.[42] The alkaline hydrolysis of 3-methyllumiflavin and **12b** are catalyzed by specific but not by general base catalysis. In the case of flavins, hydrolysis occurs by a path first order in HO^- which involves nucleophilic attack at C^{10a} (corresponding to C^6 of **12b**) and by a path which is second order in [HO^-] and involves attack at C^4 (corresponding to C^4 in **12b**) (see eq 18).[58] The hydrolysis of amides is often



(18)

found to be second order in hydroxide ion.[59] In the alkaline pH range, **12b** is also hydrolyzed via two pathways which are first and second order in hydroxide ion (Figure 3, eq 9). The hydrolysis of 3-methylalloxane, which exists in aqueous solution as a hydrate similar in structure to the C^5 HO^- adduct of **12b** (i.e., **12b**HO^-), has been shown to possess (above a pH of 5) a log k_{ly} vs. pH profile much like that of **12b**.[42a] By use of ^{14}C labeling experiments Kwart and co-workers established the nucleophilic attack to be at the equivalent C^4 and C^6 positions (eq 19).[42b]

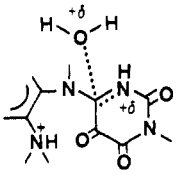
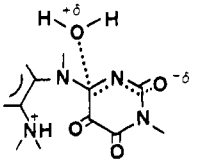
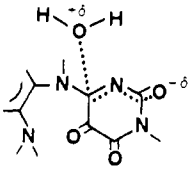
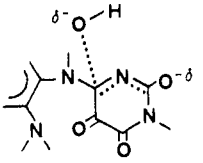
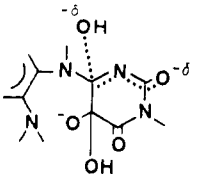


(19)

In contrast to flavins and to 3-methylalloxane, which are stable in the acidic pH range, **12b** proved to be most stable in the neutral pH range (see Figure 3), but showed lyate catalyzed hydrolysis over the whole pH range investigated. A product of hydrolysis at all pH values is *N*,*N*′,*N*′,4,5-pentamethyl-*o*-phenylenediamine (**1c**). Nucleophilic addition of lyate species to the C^6 position (in analogy to hydrolysis of flavins by attack at C^{10a} and alloxane

(56) Hine, J.; Koser, G. F. *J. Org. Chem.* **1971**, *36*, 1348–1351.
(57) Charton, M. *J. Am. Chem. Soc.* **1964**, *29*, 1222–1227.
(58) Smith, S. H.; Bruice, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 2875–2881.
(59) Bruice, T. C.; Benkovic, S. J. "Bioorganic Mechanisms"; Benjamin: New York, 1966.

Chart I

KINETIC TERMS (OF EQ. 9)	POSSIBLE CRITICAL TRANSITION STATES
$k^{H}_{+a_H}[12bH^+]$	
$k^{H}[12bH^+]$	
$k^{H_2O}[12b]$	
$k^{HO^-}[HO^-][12b]$	
$k_-^{HO^-}[HO^-][12bOH^-]$	

by nucleophilic attack at C^6 or C^4, as in eq 18 and 19) of **12b** and its protonated species as well as its C^4-hydroxyl adduct explains both the pH dependence of the kinetics of hydrolysis of **12b** and the formation of **1c** as a product at all pH values (Chart I). The kinetic term $k[HO^-]^2[\mathbf{12b}]$ cannot relate to the hydrolysis of **12b** per se since **12b** exists as its hydrate anion **12bHO⁻** in the pH range at which hydrolysis becomes dependent upon $[HO^-]^2$. For this reason a reaction second order in $[HO^-]$ and first order in **12b** would appear kinetically as first order in $[HO^-]$.

The electrochemical investigation of the redox properties of 12b and its reduced form 13b has been carried out only in aprotic solution, due to the instability of **12b** in protic solvents and to the insolubility of **13b** in neutral aqueous buffers. The reduction of **12b** in acetonitrile is best compared to the reduction of benzoquinones,[60] riboflavin, and 3-methyllumiflavin[61] in aprotic solvents, for which electrochemical data are available. Only electrochemistry in aqueous solution has been done with the related redox system of alloxane (cf. formulas in Scheme III).[25] The oxidation of the reduced form **13b** in acetonitrile can be compared with the corresponding electrochemical oxidation of hydroquinones.[62]

Unlike (substituted) benzoquinones,[60] which provide two reversible one electron reduction waves (e.g.,2,6-dimethyl-*p*-benzoquinone, which has E_m values at ca. −0.66 and −1.09 V; calculated from ref 60 by adding 0.242 V to adjust to NHE), compound **12b** gives only one quasi-reversible peak at −0.42 V, followed by another very broad reduction wave, whose shape and size strongly depend on the concentration and the scan rate. A one-electron reduction wave ($E_{1/2}$ = −0.53 V), followed by two further reductions ($E_{1/2}$ = −0.78 and −1.25 V), whose potential and limiting current depended on the scan rate and the concentrations, was found for the polarography of riboflavin in Me_2SO.[61] This behavior was tentatively interpreted as the result of reactions (either protonations with residual water or dimerizations etc.) of the intermediate semiquinone. We think that the same explanation may be applicable in our case. One of the possible reactions of an intermediate semiquinone of **12b** could be dimerization to an alloxantin (cf. formula IVd in Scheme III) like compound. Regardless of the particular interpretation, electrochemical reduction of **12b** has shown that its redox behavior is similar to oxidized flavins under analogous conditions. The oxidation of the reduced **13b** gives an irreversible two-electron wave with a peak potential at −0.58 V. Irreversible two-electron oxidation waves have been observed for hydroquinones (e.g., at +1.36 V for benzohydroquinone),[62] far from the reversible reduction potentials of their oxidized forms.

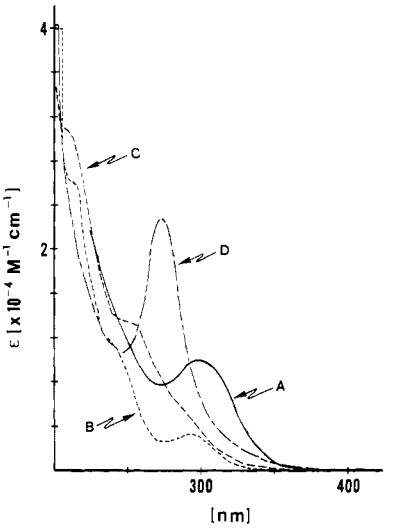


Figure 7. Final UV/vis spectra of a 5 × 10^{-5} M solution of **12b** after reduction in 0.2 M anaerobic hydrazine hydrochloride buffer (pH 7.58, μ = 1 with KCl, 1% CH_3CN) (A), after hydrolysis at pH 6.46 (see Figure 1) (B), and after methanolysis (see Figure 1) (C) and the final spectrum after hydrolysis of **5c** (5 × 10^{-5} M) in 0.02 M phosphate buffer (pH 6.46, μ = 1 with KCl, 1% CH_3CN) (D).

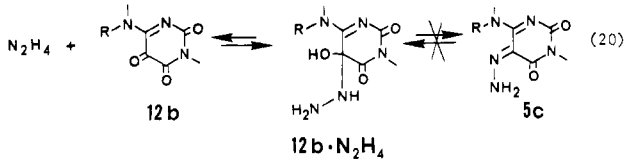
The reduction of 12b by hydrazine buffers (cf. Figure 4, trace A) proved to be fast enough to be competitive with hydrolysis and could therefore be studied in aqueous solution. This result is comparable to analogous reactions of alloxane, whose fast reduction by phenylhydrazine had been observed as early as 1887.[63] In contrast to alloxane and **12b**, the reaction of phenylhydrazine with flavins in aqueous solution is rather slow.[64]

The end product of anaerobic hydrazine reduction of **12b** (curve A in Figure 7) can be distinguished from the products of its hydrolysis (curve B) and methanolysis (curve C). The UV/vis spectrum of the product formed on reaction of **12b** with hydrazine in water is identical with that of the isolated product **13b** obtained by dithionite reduction of **12b**. That the reduction of **12b** by hydrazine does not proceed through the formation of the C^5-hydrazone of **12b** (i.e., **5c**) is shown by the following experiments. Hydrolysis of the hydrazone **5c** (pH 6.46 under N_2) is characterized by its pseudo-first-order disappearance (300 and 223 nm) with isosbestic points at 254, 285, and 215 nm. The spectrum of the hydrolysis products is shown as line D in Figure 7. It was found that the spectrum of the products produced from **5c** in hydrazine buffers corresponded to the hydrolytic products of **5c**. Thus, **5c** is not reduced by hydrazine. The hydrazone **5c** therefore cannot be on the reaction path leading from **12b** to **13c**, nor can

(60) Ryba, O.; Pilar, J.; Petranek, J. *Collect. Czech. Chem. Commun.* **1968**, 2581–2588.
(61) Tatwawadi, S. V.; Santhanam, K. S. V.; Bard, A. *J. Electroanal. Chem. Interfacial Electrochem.* **1968**, *17*, 411–419.
(62) (a) Eggins, B. R.; Chambers, J. Q. *J. Chem. Soc., Chem. Commun.* **1969**, 232–233. (b) Mann, C. K.; Barnes, K. K. "Electrochemical Reactions in Nonaqueous Systems"; Dekker: New York, 1970.
(63) Pellizzari, G. *Gazz. Chim. Ital.* **1887**, *17*, 254.
(64) Nagy, J.; Kenney, W. C.; Singer, T. P. *J. Biol. Chem.* **1979**, *254*, 2684–2688.

5c be in equilibrium with any species on the reaction path.

Our kinetics of the anaerobic reduction of **12b** (see Results) have shown that the transition state leading to the reduction of **12b** is composed only of the species **12b** and free hydrazine but not of any (specific or general) acid or base catalysts. Furthermore, the dependence of the initial absorbance of **12b** on the concentration of free hydrazine demonstrated the rapid formation of a preequilibrium between **12b** and an adduct or complex of **12b** and hydrazine. In analogy to alloxane hydrate (IVe in Scheme III), and to the structure of our isolated methanol adduct **14b** (see Scheme VII), it is reasonable to assume that hydrazine adds to the C^5-carbon of **12b** to provide the carbinolamine, eq 20. The

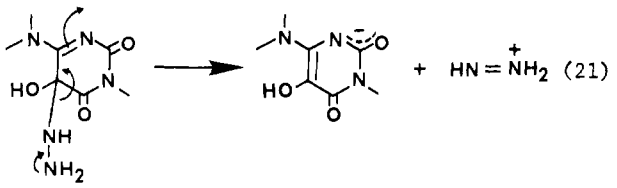


(20)

rather large decrease in the initial absorption (342 nm) of **12b** in hydrazine-buffered solutions (ca. 80% over a concentration change in N_2H_4 of 1 order of magnitude above pH 9.0) speaks for a covalent adduct rather than a charge-transfer complex.

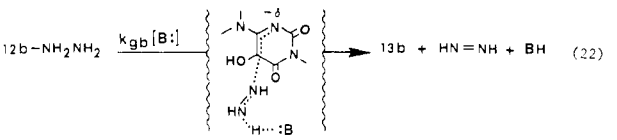
As **12b** is a two-electron acceptor, capable of reacting via an intermediate radical (see interpretation of the electrochemical data), and stepwise one-electron oxidation of hydrazines is well-known,[65] two possible mechanisms for the two-electron reduction of **12b** to **13b** must be considered: (i) concerted two-electron transfer, which could go via a covalent intermediate, a hydride transfer, etc., and (ii) stepwise transfer of one-electron equivalents (electrons or hydrogen atoms). The possibility of a concerted two-electron transfer via a covalent intermediate (which could be **12b**·N_2H_4 as formulated in eq 20) is of some interest because of the covalent interactions postulated for copper-dependent amine oxidases (cf. Scheme II).

The formation of **13b** by spontaneous decomposition of **12b**·N_2H_4 would, without base catalysis, produce a protonated diazine (eq 21) whose pK_a should be less than zero (compare to pK_a values



(21)

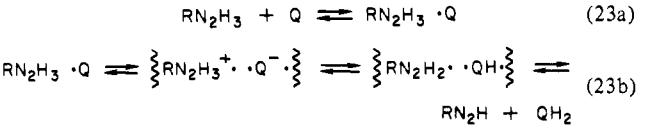
of phenyl-substituted azo compounds).[65] To obviate the formation of this unstable species, a concerted reaction which involves general base removal of a nitrogen-bonded proton would be expected (eq 22). The kinetic results establish the absence of either specific



(22)

or general base catalysis in the hydrazine reduction of **12b** to **13b**. It is possible that the mechanism of eq 22 is in effect with H_2O acting as the general base catalyst. This would be possible in the pH range investigated if the Bronstead β value was quite small ($\sim$0.2) corresponding to an early transition state.

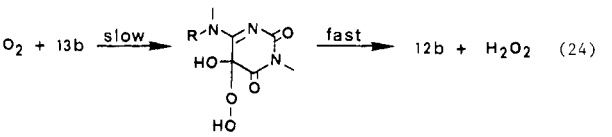
An alternative to the two-electron transfer via a covalent intermediate would be a one-electron transfer (either electron or hydrogen atom, or $1e^- + H^+$) within a charge transfer complex as discussed for the oxidation of phenylhydrazines by benzoquinones (see eq 23).[66] A stepwise electron transfer was formulated on the basis of the detection of CIDNP signals of the hydrazine during the course of the reaction. It is clear that a definite assignment of a mechanism to the reduction of **12b** by hydrazine requires further work.



(23a)

(23b)

The oxidation of 13b in acetonitrile does not show any evidence for an intermediate between the reduced form **13b** and the oxidized form **12b**. Thus, the reaction follows the first-order rate law in the presence of excess O_2. This would be consistent with a reaction mechanism involving a C^5-hydroperoxide intermediate (eq 24).



(24)

In contrast, the reaction in water shows complicated autocatalytic behavior. This observation may have been foretold, given the relationship of **13b** to dialuric acid (IVb in Scheme III) and dihydroflavin. The reduced semiquinone of **12b** is presumably the autocatalytic agent in the reaction of **12b** with dioxygen in aqueous solution. Semiquinones of alloxane[67] and flavin[68] play an important role in the autoxidation of these compounds.

Conclusions of biochemical relevance stem from the synthesis and study of **12b** which, in structure, closely resembles the intermediate postulated in mechanisms offered for flavoenzyme mono- and dioxygenases[4,18,21] and as a cofactor in the copper-requiring plasma amine oxidase.[22] Also, as a 6-amino-5-oxo-3*H*,5*H*-uracil, **12b** resembles the postulated intermediate derived from biopterin in a proposed mechanism for phenylalanine hydroxylase.[16a-f]

Compound II of the hypothetical flavin monooxygenase mechanism of Scheme I, like **12b**, contains phenylenediamine (reducing agent) covalently bound to an alloxane moiety (an oxidizing agent). It has been aruged that the mechanism of Scheme I cannot be correct because formation of compound II (or its precursor I) would be followed by its self-destruction via intramolecular oxidoreduction.[69] The finding that **12b** does not undergo an intramolecular oxidation–reduction reaction negates this criticism of the mechanisms of Scheme I. The argument that 4a,5 ring opening does not occur (because of intramolecular oxidation–reduction) but that the hydroperoxy function must migrate to another position (9a, 10a, etc.) prior to a "safe activation" by ring opening has no basis. (Other arguments against this proposal have been offered from this laboratory[70]). The only indication of an interaction of the phenylenediamine–alloxane halves of **12b** is its light absorption which reaches to $\sim$550 nm. This feature can be ascribed to a charge transfer interaction.

The synthesis of **12b**, through the intermediate **10b**, does indicate that protonation of the N^5 position of the flavin 4a-pseudobase (5-HFl-4a-OH) could result in 4a–5 bond scission of the 4a,5-dihydroisoalloxazine ring. This finding not only bears upon the proposed mechanisms of Schemes I and II but could be important in future deliberations on flavin and flavoenzyme reactions. Though we have yet to investigate the reactions of **12b** with amines, the rapid reduction of **12b** by hydrazine in aqueous solution may be in accord with the proposed role of 4a,5 ring opened flavin in

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(70) (a) Miller, A.; Bruice, T. C. *J. Chem. Soc., Chem. Commun.* **1979**, 896–897. (b) Miller, A.; Bruice, T. C. *Ibid.* **1980**, 693–694.

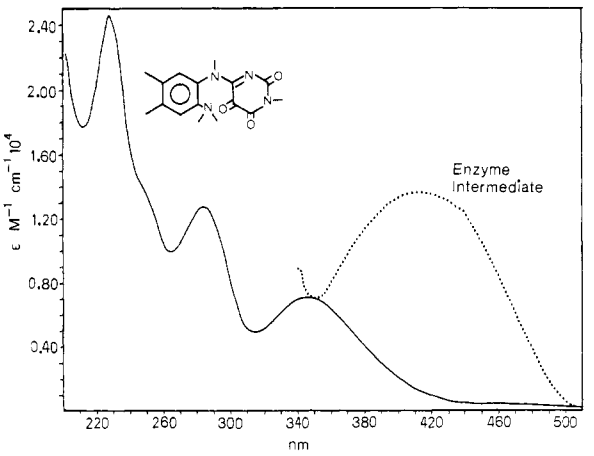
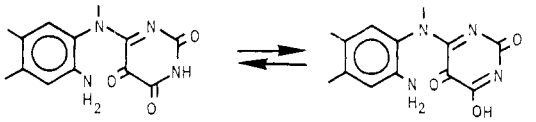


Figure 8. Comparison of the UV/vis spectra of **12b** in acetonitrile and of the enzyme-bound intermediate proposed to be compound II.

the copper-containing amine oxidase as outlined in Scheme II.

The rather positive comments of the last paragraph must be tempered by the finding that the UV/vis spectrum of **12b** in acetonitrile, water, and methanol does not resemble that of the enzyme-bound intermediate of *p*-hydroxybenzoate hydroxylase which has been assigned[21] the structure II of Scheme I. The spectrum of **12b** (in acetonitrile) is compared to that of the enzyme-bound intermediate in Figure 8. Inspection of Figure 8 reveals that the λ_{max} of **12b** is ~60 nm to the blue when compared to the enzyme intermediate and, most importantly, the extinction of **12b** is but 7120 cm^{-1} M^{-1} while that of the enzyme intermediate is minimally 15 000 cm^{-1} M^{-1}. Unless compound II of Scheme I is bound to the apoprotein in a rather peculiar manner so that its λ_{max} and ϵ are greatly changed, one can dismiss the mechanisms a/b or c of Scheme I. In this regard it should be noted that reduced and oxidized flavins and flavin derivatives when enzyme bound and free in solution possess similar spectra. There remains the possibility that the enzyme-bound intermediate is an *o*- or *p*-quinoid tautomer of compound II in Scheme I (eq 25). The



(25)

o- or *p*-quinoid tautomer is expected to be less stable than the oxo-3*H*,5*H*-uracil form and, therefore, less probable. Synthetic approaches to test this possibility are, nonetheless, under way.

Yamada and Yasunobu[71] have determined the UV/vis spectrum of the unknown cofactor of plasma amine oxidase [λ_{max} = 380 nm, without bound Cu(II)] as well as the spectra of the enzyme-bound hydrazone derivative [λ_{max} ~ 300 nm, without Cu(II)]. Extinction coefficients were not provided. The λ_{max} for **12b** is ~40 nm blue shifted when compared to the enzyme-bound cofactor, whereas the hydrazone of **12b** (i.e., **5c**) possesses a λ_{max} identical to that of the enzyme-bound hydrazone derivative of the cofactor.

Acknowledgment. This work was supported by a grant from the National Institutes of Health. We thank Professors S. J. Benkovic and J. J. Villafranca for performing ^{13}C NMR measurements and Dr. Michael W. Nee of this laboratory for collecting and interpreting the ^{15}N NMR data.

Registry No. 1c, 85894-78-6; **2**, 878-86-4; **3a**, 947-54-6; **3b**, 58758-67-1; **3c**, 79872-70-1; **3c**·HCl, 85908-83-4; **4a**, 79854-10-7; **4b**, 65357-96-2; **4c**, 79854-11-8; **5a**, 79854-12-9; **5b**, 79854-13-0; **5b′**, 85894-79-7; **5c**, 79854-14-1; **6**, 85894-80-0; **7**, 55832-86-5; **8a**, 37047-12-4; **8b**, 4074-59-3; **9**, 18636-32-3; **10a**, 26257-55-6; **10b**, 40815-89-2; **11a**, 79854-09-4; **12b**, 79854-08-3; **13b**, 85894-81-1; **14b**, 85894-82-2; *N*-formyl-*N*,*N*′,*N*′,4,5-pentamethyl-*o*-phenylenediamine, 54929-07-6; flavin monooxygenase, 9038-14-6; pterin monooxygenase, 9029-73-6.

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