Solution Structures of 6,7-Dimethyl-8-substituted-lumazines. ^{13}C NMR Evidence for Intramolecular Ether Formation†

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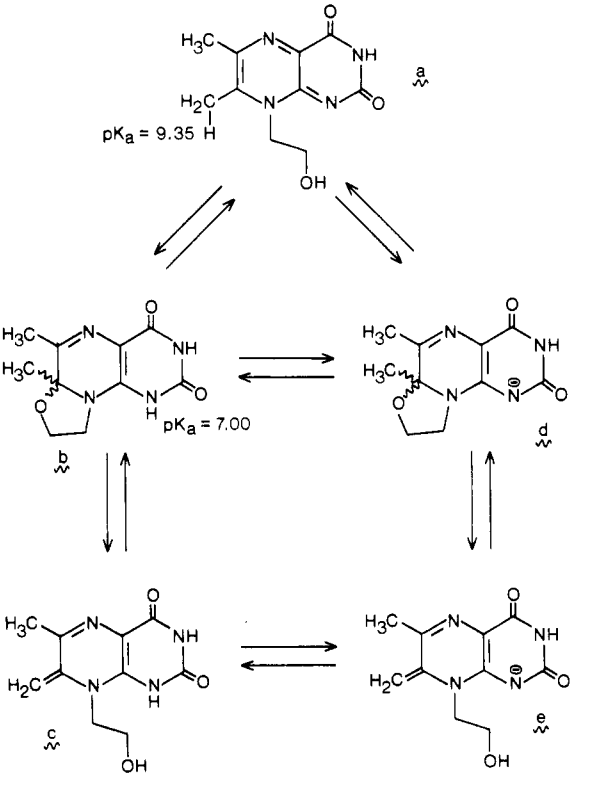
6,7-Dimethyl-8-substituted-lumazines were shown to exist in alkaline solution (pH 11, sodium phosphate buffer), as mixtures of intramolecular ethers and 7α exo methylene anions as observed by high-field NMR spectroscopy. In the case of 6,7-dimethyl-8-(D-ribityl)lumazine the ^1H and ^{13}C NMR spectra revealed five major anionic species in basic solution. The structures were determined to be the 7α exo methylene anion (17%), diastereomeric intramolecular five-membered ring ether anions (2′ oxygen covalently bonded to C7, 40% and 22%), and diastereomeric intramolecular six-membered ring ether anions (3′ oxygen covalently bonded to C7, 14% and 7%). Lumazines with a D-arabityl, D-xylityl, and D,L-glyceritYl side chain also proved to exist as five major equilibrating species in basic aqueous solution. Only diastereomeric intramolecular six-membered ring ether (16% and 5%) and 7α exo methylene (79%) anions of 6,7-dimethyl-8-(2-deoxy-D-ribityl)lumazine were observed at pH 11. Diastereomeric five-membered ring ether (45% and 39%) and 7α exo methylene (16%) anions were observed at equilibrium for 6,7-dimethyl-8-(D,L-2-hydroxypropyl)lumazine. A series of 8-(ω-hydroxyalkyl)lumazines with the side chain varying in length from two to six carbon atoms was also analyzed. In all these cases, the 7α exo methylene anion is the major component of the alkaline equilibrium mixture. The formation of five-, six-, and seven-membered ring ether anions was observed in the 8-(ω-hydroxyalkyl)lumazine series. Only one anionic lumazine, the 7α exo methylene species, was detected for the 8-(5-hydroxypentyl), 8-(6-hydroxyhexyl), and 8-methyl analogues. The ^1H and ^{13}C NMR data give no support for the direct hydration of lumazines by a water molecule in neutral or basic solution.

8-Substituted derivatives of 6,7-dimethyllumazine have attracted attention since the discovery of the 8-ribityl compound as an intermediate in the biosynthesis of riboflavin. Lumazine (pK_a = 7.95) and 6,7-dimethyllumazine (pK_a = 8.40)[1] are only slightly more acidic in aqueous solution than the corresponding 8-methyllumazines (pK_a = 9.82 and 9.90, respectively).[2] The acidity of the unsubstituted parent lumazine is due to deprotonation of N1. The weaker acidity of 8-methyllumazine has been rationalized in terms of the formation of a covalent hydrate by the addition of a water molecule to the C7-N8 bond. In general, a variety of nucleophilic reactions of lumazines have been reported to take place at C7 of the pyrazine ring.[3] Hydration of heterocyclic systems is not unique to lumazines. Numerous cross-conjugated π systems, including pteridines, have been reported to form hydrated compounds.[4-6]

The titration of the hydrated species of 7-methyl-8-substituted-lumazines in basic solution is accompanied by deprotonation and the formation of 7α exo methylene anions. The hydration of 6,7,8-trimethyllumazine was originally interpreted by Pfleiderer[2] as ring opening of the transient hydrate on the basis of pK_a and UV–vis[25] spectroscopic data. However, the ^1H NMR spectrum revealed no evidence for the ring-opened lumazine and the data were reinterpreted as the formation of the 7α exo methylene anion.[7] Despite the efforts many groups[7-11] there is little evidence for the formation of the covalent hydrate of 6,7,8-trimethyllumazine. It appears that a systematic quantitative study comparing the effects of the formation of 7α exo methylene species, hydrates by water, and intramolecular ethers on the stability of lumazine anions does not exist in the literature.

Scheme I



When the substituent in the 8 position of lumazine bears a hydroxyl group, intramolecular ether formation can oc-

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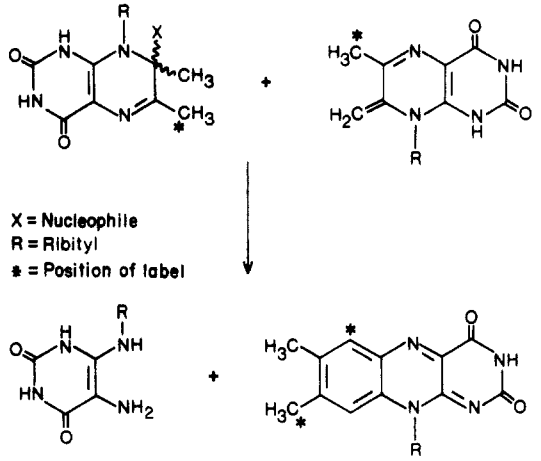
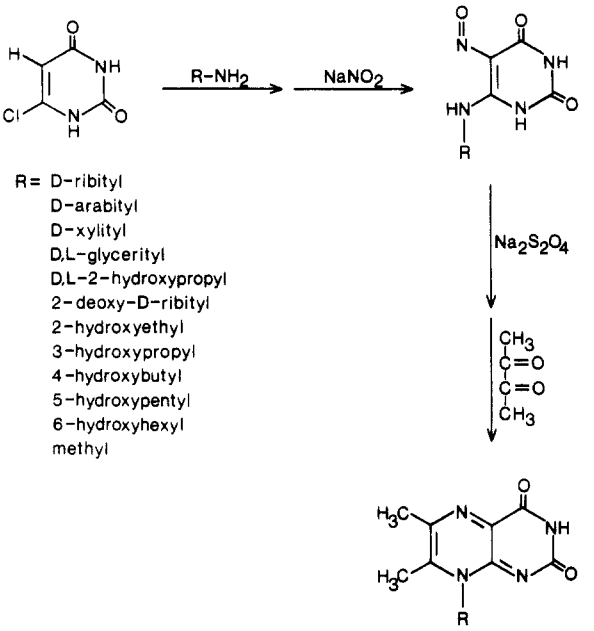
Scheme II



Scheme III



cur. The five-membered ring ether anion of 6,7-dimethyl-8-(2-hydroxyethyl)lumazine (d) (Scheme I) was proposed by Pfleiderer and co-workers[2] to explain the pH-dependent UV–vis spectra. Rapid spectroscopic measurement allowed determination of the pK_a of the isomeric lumazines (b and c). The ratio of a:(b + c) was determined by Pfleiderer[2] to be 220. Plaut confirmed the formation of the cyclic ether anion d by ^{1}H NMR and determined the ratio of d:e to be 1:2. A stable intramolecular ether of 6,7-diisopropyl-8-(2-hydroxyethyl)lumazine was isolated as colorless needles, with no detectable formation of the acyclic compound with an extended side chain.[2]

The 8-(3-hydroxypropyl) analogue has been reported by Beach and Plaut[8] to exist in alkaline solution entirely as the 7α exo methylene anion. They also report no formation of six-membered ring intramolecular ethers of 6,7-dimethyl-8-(2-deoxy-D-ribityl)lumazine.[8] The intramolecular ethers of 6,7-dimethyl-8-(D-ribityl)lumazine were initially speculated to be six-membered rings on the basis of UV data.[2] After ^{1}H NMR data became available, the structure of the ether was revised as only five membered ring anions.[7,8,12] The ratio of cyclic ether to exo methylene anions was determined by Plaut to be 3:1 on the basis of integration of the ^{1}H signals for H6α. Closer examination of the original 60-MHz ^{1}H NMR spectra of Plaut suggests that at least three species are present in an alkaline solution of 6,7-dimethyl-8-D-ribityllumazine. The circular dichroism spectra of the 8-(D-ribityl)-, 8-(D-arabityl)-, and 8-(D-xylityl) compounds in basic aqueous solution are complex and not very useful for structure determination.[13]

A reinvestigation of Plaut's report[8] that six-membered ring ether anions do not exist in basic solution has now been conducted. Such a study is biochemically relevant as well as significant in terms of understanding the chemistry of 8-substituted lumazines. The final step of the biosynthesis of vitamin B_2 involves the dismutation of two molecules of 6,7-dimethyl-8-(D-ribityl)lumazine yielding one molecule of riboflavin (Scheme II). The regiochemistry of this enzymatic reaction which is catalyzed by riboflavin synthase has been explained by invoking two isomers of the lumazine.[14-17] Formation of a dihydropyrazine as an enzyme-bound substrate at C7, a cyclic ether, or a hydrate provides an electrophilic center at C6 of the donor lumazine. Nucleophilic attack of 7α of an exo methylene isomer of the acceptor lumazine on C6 of the donor molecule would give the observed regiochemistry. The same dismutation reaction of two lumazine molecules forming riboflavin occurs nonenzymatically under neutral[18,19] and acidic conditions.[20] As part of investigations on the biosynthesis of riboflavin, we have analyzed various ^{13}C-enriched lumazine samples.[21] In addition to the desired biochemical information, the ^{13}C NMR analyses provided a technique for determining the precise composition of the lumazine in alkaline solution. Five major anionic species were detected by ^{13}C NMR in aqueous solution buffered at pH 11. Since only two anionic lumazine species were originally proposed by Plaut,[8] we decided to determine the chemical structures and equilibrium ratios of 6,7-dimethyl-8-(D-ribityl)lumazine and related compounds (Scheme III) in basic solution.

Experimental Section

Materials. All chemicals were reagent grade and were used without further purification. Cation (AG 50W-X8, 200–400 mesh) and anion (AG 1-X8, 200–400 mesh) exchange resins were from Bio-Rad. 4-Amino-1-butanol, 5-amino-1-pentanol, 6-amino-1-hexanol, D,L-1-amino-2-propanol, and 2,3-butanedione were purchased from Aldrich. The following 6,7-dimethyl-8-substituted-lumazines were prepared according to knonw procedures (see Scheme III): 8-methyl-8-(2-hydroxyethyl)-, 8-(3-hydroxypropyl)-, 8-(D,L-glyceryl)-, 8-(D-ribityl)-, 8-(D-arabityl)-, 8-(D-

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Table I. UV-Visible Absorption Maxima (λ in nm) and Extinction Coefficients (ε in L/mol-cM)

	pH	200	224 (sh)[a]	238	311	350
		A. 6-(Alkylamino)-5-amino-2,4-(1*H*,3*H*)pyrimidinediones				
4′-hydroxybutyl	5.0	26 500	17 000		14 500	500
	8.0	[500 nm (ε 110)]		5500	18 500	1000
	12.0			4000	18 500	2500
5′-hydroxypentyl	5.0	27 500	18 000		13 000	500
	8.0	[500 nm (ε 115)]		5500	17 000	1000
	12.0			4000	17 000	2000
6′-hydroxyhexyl	5.0	29 000	16 500		12 000	1000
	8.0	[500 nm (ε 130)]		5500	16 500	1000
	12.0			4000	16 500	2000
2′-hydroxypropyl	5.0	27 000	16 000		15 000	1000
	8.0	[500 nm (ε 100)]		6000	18 000	1000
	12.0			4000	18 000	2000

	pH	228	256	278	313	407
		B. 8-Alkyl-6,7-dimethyllumazines				
4′-hydroxybutyl	5.0	6000	15 000	12 000	600	12 500
	8.0	9500	17 000	13 500	2000	12 000
	12.0	29 000	16 000[b]	10 000[c]	17 000	4000[d]
5′-hydroxypentyl	5.0	7000	17 000	13 000	550	14 000
	8.0	10 000	17 500	14 000	2000	13 000
	12.0		22 000[b]	11 000[c]	21 500	6500[d]
6′-hydroxyhexyl	5.0	7000	14 000	11 000	1000	11 000
	8.0	10 000	16 000	13 000	2000	11 000
	12.0		19 000[b]	10 000[c]	18 500	6000[d]
2′-hydroxypropyl	5.0	6500	16 000	11 500	1000	13 000
	8.0	10 500	16 000	12 500	3500	10 500
	12.0	32 500		14 500	12 000	1000

[a] Shoulder. [b] 245 nm. [c] 265 nm (sh)[a]. [d] 370 nm.

xylityl)-, and 8-(2-deoxy-D-ribityl)-6,7-dimethyllumazine.[12,22,23] The ^{13}C-enriched 6,7-dimethyl-8-(D-ribityl)lumazine samples labeled at C6α, C6, C7, and C7α were prepared chemically from the appropriately labeled monooxime of 2,3-butanedione and 6-(D-ribitylamino)-5-amino-2,4(1*H*,3*H*)-pyrimidinedione (H. Sedlmaier and A. Bacher, unpublished experiments). The biosynthetically enriched samples were isolated from *Bacillus subtilis* fermentations incorporating [1-^{13}C]ribose and [1,3-$^{13}C_2$]glycerol.[21]

General Methods. UV–vis spectra were routinely recorded in 1.0-cm cells in 0.5 M sodium phosphate buffer on a Varian DMS-90 spectrophotometer. The extinction coefficients of the absorption maxima at pH 5, 8, and 12 for the previously unreported lumazines and nitrosopyrimidinediones are given in Table I. The temperature of the cell holder was maintained by a Haake Model D1 constant temperature circulating water bath. The pK_a values of the lumazines were calculated from UV ($1.0–2.0 \times 10^{-5}$ M) and ^1H NMR (0.03–0.04 M) titration data. The acidity constant is the inverse of the intercept of a double reciprocal plot fitted to a straight line. Linear regression analyses were performed on a programmable Texas Instruments Model 59 calculator. Yields are of isolated recrystallized products dried (0.05 torr) to constant weight. Melting points are uncorrected and were determined with Mel-temp apparatus in capillary tubes. Elemental analyses were performed by the Analytical Laboratory of the Technische Universität München.

NMR Measurements. The ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were measured on a Bruker WM 300 spectrometer in aqueous sodium phosphate buffer (0.50 M, 0.5 mL of H_2O/D_2O, 8:2 (v/v). Solvent suppression in ^1H NMR spectra was accomplished by presaturation of the water resonance. At alkaline pH the polyhydroxylated lumazines were very soluble allowing the analysis of more concentrated (0.4 M) solutions. The ^{13}C NMR spectra of the monohydroxyalkyl lumazines (0.2 M) were measured in lower sodium phosphate buffer concentration (0.10 M) to improve the solubility. The ^{13}C NMR spectra were recorded by using a 60° pulse, 2.0-s repetition time, continuous ^1H broadband decoupling, 32K data sets and 1.0-Hz line broadening. Internal TSP was used as the chemical shift reference for all the NMR spectra recorded in aqueous solutions. The spectra recorded in Me_2SO-d_6 are referenced to the residual solvent signals at δ 2.49 for ^1H and δ 39.5 for ^{13}C. The chemical shifts are given in ppm (δ), and ^1H multiplicities are designated as s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), and m (multiplet).

6-[(4-Hydroxybutyl)amino]-5-nitroso-2,4(1*H*,3*H*)-pyrimidinedione. Condensation of 4-amino-1-butanol with 6-chloro-2,4(1*H*,3*H*)-pyrimidinedione followed by nitrosation was conducted as described in the literature for the ribityl derivative.[23] The product was purified by anion exchange chromatography (AG 1X8, formate) and recrystallized from water, yielding an red-orange solid (61%, mp 201–203 °C, dec).

Similarly 5-amino-1-pentanol gave the next higher homologue (pink-orange solid, 42%, mp 212–214 °C, dec). The 6-[(6-hydroxyhexyl)amino]-5-nitrosopyrimidine was likewise prepared as an orange solid (mp 182–184 °C, dec) in 45% yield. Condensation of D,L-1-amino-2-propanol with the chloropyrimidine followed by nitrosation gave 6-[(D,L-2-hydroxypropyl)amino]-5-nitrosopyrimidinedione in 41% yield isolated as a red-purple solid (mp 202–204 °C dec).

6,7-Dimethyl-8-(4-hydroxybutyl)lumazine and Analogues. Reduction of the nitrosopyrimidine prepared above with sodium hydrosulfite followed by condensation with diacetyl proceeded smoothly as outlined by Plaut and Harvey.[23] The desired product was purified by applying the crude reaction mixture to a AG 50 W-X8 (H^+) cation exchange column and eluting the fluorescent material with water. Recrystallization from ethanol/water (1:1) gave yellow plates (mp 237–239 °C) in 88% yield: ^1H NMR (Me_2SO-d_6) 8.03 (s, 1 H), 4.52 (t, exchanges in D_2O, 1 H), 4.43 (t, 2 H), 3.69 (t, 2 H), 2.64 (s, 3 H), 2.48 (s, 3 H), 1.73 (m, 2 H), 1.53 (p, 2 H); ^{13}C NMR (Me_2SO-d_6) 160.6, 155.3, 150.5, 146.6, 139.6, 131.9, 60.2, 47.5, 29.4, 23.5, 21.5, 17.0. Anal. Calcd for $C_{12}H_{16}N_4O_3$: C, 54.53; H, 6.10; N, 21.20. Found: C, 54.55; H, 6.09; N, 21.19.

6,7-Dimethyl-8-(5-hydroxypentyl)lumazine was prepared as outlined above as a yellow crystalline solid (82%, mp 254–256 °C dec): ^1H NMR (Me_2SO-d_6) 8.02 (s, 1 H), 4.41 (d of d, 2 H), 4.41 (t, exchanges in D_2O, 1 H) 3.40 (t, 2 H), 2.64 (s, 3 H), 2.48 (s, 3 H), 1.69 (p, 2 H), 1.47 (m, 2 H), 1.44 (m, 2 H); ^{13}C NMR (Me_2SO-d_6) 160.6, 155.3, 150.5, 146.5, 139.5, 132.0, 60.4, 47.6, 31.9, 26.3, 22.8, 21.5, 16.9. Anal. Calcd for $C_{13}H_{18}N_4O_3$: C, 56.10; H, 6.52; N, 20.13. Found: C, 56.22; H, 6.52; N, 20.09.

The 8-(6-hydroxyhexyl) analogue was isolated in 68% yield as an orange solid (mp 236–238 °C dec): ^1H NMR (Me_2SO-d_6) 8.03 (s, 1 H), 4.39 (dd, 2 H), 4.37 (t, exchanges in D_2O, 1 H), 3.62 (t,

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Table II. Equilibrium Distributions[a] of Anionic Compounds of 6,7-Dimethyl-8-substituted-lumazines

substituent	pK^b	7α-CH_2	2′-ethers[c]	3′-ethers[c]
D-ribityl	8.31[d]	17	40:22	14:7
D-arabityl	8.29	16	30:13	24:17
D-xylityl	8.33	16	35:22	14:13
D,L-glyceryityl	8.66	27	40:21	8:4
2′-hydroxypropyl	8.90	16	45:39	
2′-hydroxyethyl	9.32[e]	59	41	
2′-deoxyribityl	9.52	79		16:5
3′-hydroxypropyl	9.72	89		11
4′-hydroxybutyl	9.84	69		31[f]
5′-hydroxypentyl	10.02	100		
6′-hydroxyhexyl	9.97	100		
methyl	9.93[g]	100		

[a] Ratios are in percent determined by ^1H NMR at 25 °C. [b] Average of ^1H NMR and UV–vis titration data at 25 °C. [c] Diastereomeric assignments are arbitrary and may be reversed. [d] 8.29, Pfleiderer et al. (1966). [e] 9.35, Pfleiderer et al. (1966). [f] 4′-Ether. [g] 9.90, Pfleiderer et al. (1966); 9.85, McAndless and Stewart (1970).

2 H), 2.64 (s, 3 H), 2.47 (s, 3 H), 1.68 (p, 2 H), 1.43 (m, 2 H), 1.36 (m, 4 H); ^{13}C NMR (Me_2SO-d_6) 160.6, 155.3, 150.5, 146.5, 139.6, 131.9, 60.5, 47.5, 32.3, 26.4, 26.1, 25.1, 21.5, 17.0. Anal. Calcd for $C_{14}H_{20}N_4O_3$: C, 57.52; H, 6.90; N, 19.17. Found: C, 57.55; H, 6.88; N, 19.04.

6,7-Dimethyl-8-(2-hydroxypropyl)lumazine was prepared in 39% yield as a yellow crystalline solid (mp 276–278 °C dec): ^1H NMR (Me_2SO-d_6) 11.04 (s, 1 H), 5.05 (d, exchanges in D_2O, 1 H), 4.57 (d of d, 1 H), 4.17 (m, 1 H), 4.16 (dd, 1 H), 2.69 (s, 3 H), 2.51 (s, 3 H), 1.21 (d, 3 H); ^{13}C NMR (Me_2SO-d_6) 160.7, 155.1, 150.8, 147.9, 139.3, 131.9, 63.1, 54.6, 21.7, 20.9, 18.0. Anal. Calcd for $C_{11}H_{14}N_4O_3$: C, 52.79; H, 5.64; N, 22.39. Found: C, 52.47; H, 5.87; N, 21.73.

Results and Discussion

Our approach to the determination of the solution composition of the anionic lumazines centers on extensive use of ^1H and ^{13}C NMR spectroscopy. Structural assignments were facilitiated by the use of chemically and enzymatically synthesized ^{13}C-enriched lumazines. Quantitative data on the equilibrium mixtures were determined by integration of ^{13}C NMR spectra. ^1H NMR data confirmed the solution composition ratios and offered additional proof of structural assignments. The ^{13}C and ^1H chemical shift data were readily compared by recording the 2D NMR chemical shift correlation spectrum. pH titrations were followed by both ^1H NMR (0.03–0.04 M) and UV–vis spectroscopy (10^{-5} M). The equilibrium pK_a values for the 7α protons are an average of the two methods (Table II).

pK_a Measurements. Spectrophotometric titrations were conducted by monitoring the absorption change at 407 nm of the neutral lumazine and 313 (and 228 nm) of the anionic lumazines. Well-defined isosbestic points at 350 ± 10 nm were observed in all the titration curves. Isosbestic points at 270 nm and 240 nm were also present, indicating that only monoanions are formed. Our pK_a measurements of the 7α protons of the 12 lumazines studied are listed in Table II. Three lumazine acidities, 8-(D-ribityl), 8-(2-hydroxyethyl), and 8-methyl, were previously known[2,9] and agree within 1% with the present measurements (Table II). In the case of 6,7,8-trimethyllumazine pK_a values for protonation of N1 (0.85), deprotonation of 7α (9.90), and deprotonation of N3 (14.11) had been reported.[2]

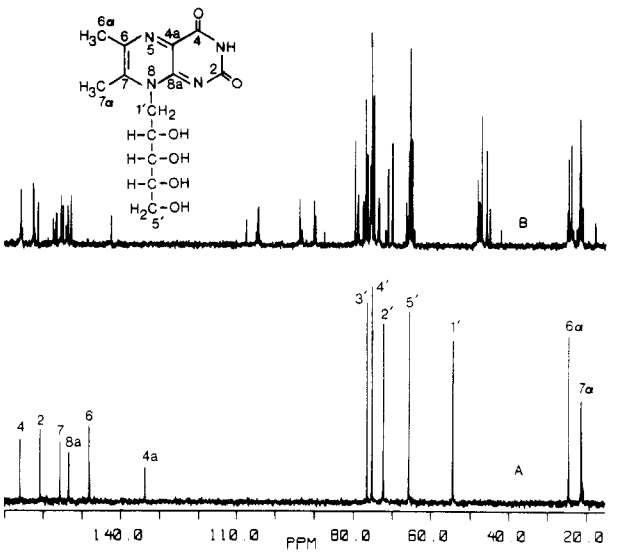

Figure 1. ^{13}C NMR spectra of 6,7-dimethyl-8-(D-ribityl)lumazine at natural abundance in 0.50 M sodium phosphate buffer solution in H_2O/D_2O at pH 7 (A) and pH 11 (B).

^{13}C NMR of 8-(D-Ribityl)-6,7-dimethyllumazine. The ^{13}C NMR spectra of the neutral 8-(D-ribityl) compound was recorded in 0.5 M sodium phosphate buffer, pH 7 (Figure 1 and Table III). In conjunction with the biosynthetic studies, the ^{13}C NMR spectrum had been previously unequivocally assigned.[21] Chemical shift assignments were based on the following criteria: deuterium exchange of protons at C7α, two bond deuterium isotope shift at C7 due to C7α CH_2D species, and 2D NMR carbon–proton correlation spectroscopy. Two signals which are well-isolated at δ 133.7 (C4a) and δ 54.3 (C1′) are significant to the subsequent discussions of anionic lumazines. The ^{13}C NMR data at pH 7 are consistent with a solution structure of 6,7-dimethyl-8-(D-ribityl)lumazine with no intramolecular ether formation (Figure 2).

Table III. ^{13}C NMR Chemical Shifts of 6,7-Dimethyl-8-(D-ribityl)lumazine

		anionic compounds				
			five-membered ring		six-membered ring	
carbon assignment	neutral compound	7α-exo methylene	major	minor	major	minor
2	160.8	161.4	162.6	162.6	162.5	162.3
4	166.8	165.4	165.7	165.9	165.8	165.0
4a	133.7	107.6	104.5	105.0	104.7	104.2
8a	153.4	156.7	155.5	155.5	157.5	157.2
6α	24.4	24.6	21.5	21.0	21.6	21.7
6	148.1	153.7	152.9	154.1	154.9	155.2
7	155.6	142.5	93.7	93.2	89.7	87.3
7α	21.2	90.0	23.7	24.8	21.8	17.8
1′	54.3	48.3	47.1	47.8	45.9	45.0
2′	72.2	71.2	76.8	78.7	70.1	66.4
3′	76.4	76.4	74.6	73.4	79.5	77.5
4′	75.1	75.1	75.3	75.6	75.2	75.4
5′	65.5	65.7	65.4	65.6	65.0	64.9

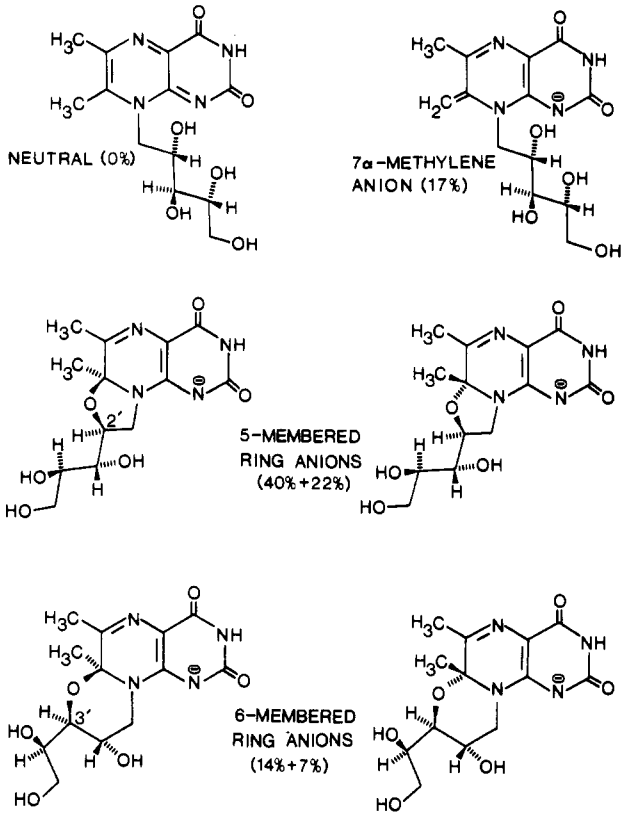

Figure 2. Structures of 6,7-dimethyl-8-(D-ribityl)lumazine in neutral and alkaline solution. Percentages are the equilibrium composition at pH 11 as determined by ^1H NMR spectroscopy.

In alkaline solutions, the ^{13}C NMR spectrum of 6,7-dimethyl-8-(D-ribityl)lumazine is considerably more complicated (Figure 1 and Table III). It is apparent, by noting the disappearance of the C1′ and C4a signals, that at pH 11 the neutral lumazine is not observed in the equilibrium mixture. The chemical shifts of C4, C4′, and C5′ are the least effected by the formation of the anionic species. Minor changes associated with the formation of the negative charge localized at N1 are observed at C2 and C8a. At pH 11, new signals appear which are not detected at pH 7, notably at δ 142.5 (1 line), δ 107 (1 line), δ 104–105 (4 lines), and δ 87–93 (5 lines). Integration of the resolved regions (i.e., δ 45–48 (C1′)) of the ^{13}C NMR spectrum suggests that five major lumazine anions are equilibrating in solution (Figure 2).

The pyrazine ring carbons 6α, 6, 7, and 7α were readily assigned by recording the ^{13}C NMR spectra of the four synthetically enriched 6,7-dimethyl-8-(D-ribityl)lumazine samples carrying the majority of the ^{13}C in C6α, C6, C7, and C7α, respectively. The enriched signals of the lumazine with the majority of the label at C6 is shown in Figure 3. Integration of the C6 and C7 resonances gave similar ratios for the five major anionic lumazine species. Analysis of the lumazine enriched primarily at C7α allowed one to assign the peak at δ 90.0 to C7α of the exo methylene anion. The 7α methyl signals at δ 23.7, 24.8, 21.8, and 17.8 are distinguished from the 6α resonances by the presence of the CH_2D species. A 0.15-ppm upfield one-bond deuterium isotope effect is observed at all five 7α signals. The 6α carbon of the exo methylene anion resonates at δ 24.6 and is well resolved from the four 6α lines of the intramolecular five-membered (δ 21.0 and 21.5) and six-membered (δ 21.6 and 21.7) ring ether anions. The integral of the well-resolved ^{13}C signal at δ 142.5 corresponds to C7 of the exo methylene anion. The chemical shifts of the remaining four lines in the δ 87–93 region are consistent

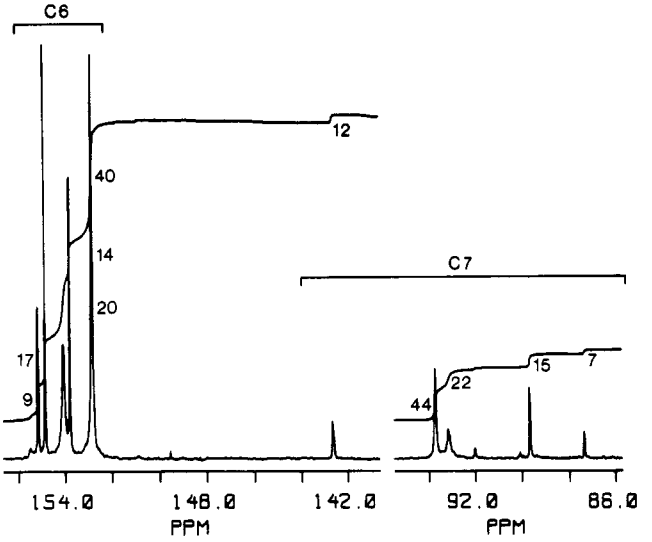

Figure 3. Segment of the ^{13}C NMR spectrum in alkaline solution (pH 11) of synthetic 6,7-dimethyl-8-(D-ribityl)lumazine enriched at C6 (72% ^{13}C) and C7 (18% ^{13}C).

with an sp^3 carbon at C7 which is bonded directly to oxygen and nitrogen.

The 4a carbons are assigned to the five lines in the δ 104–107 region, being significantly more shielded than in the neutral lumazine (δ 134). The C4a resonance of the exo methylene anionic species is the most downfield signal, δ 107.6. The four lines at δ 104–105 are assigned to C4a of the two sets of diastereomeric ether anions. Little structural information can be deduced from the chemical shifts of the C6 (δ 153–155), C8α (δ 156–158), C2 (δ 162), and C4 (δ 165) signals of the lumazine ring.

Assignment of the ribityl side chain carbon atoms was assisted by measuring the ^{13}C NMR spectra at pH 11 of the biosynthetically enriched 6,7-dimethyl-8-(D-ribityl)lumazine samples. The lumazine derived from [1-^{13}C]ribose was 26% enriched at C1′.[21] Integration of the enriched signals (Figure 4)[24] gave the same ratio of 40:22:17:14:7 as observed in the spectra of the synthetic lumazines ^{13}C-enriched at C6a, 6, 7, or 7a (Figure 3). The five 2′ carbon peaks were also assignable due to the one-bond carbon–carbon coupling to the highly enriched C1′. The biosynthetically labeled lumazine from the [1,3-$^{13}C_2$]glycerol fermentation displayed carbon–carbon coupling at both C2′ and C3′. Consequently, the C3′ resonances could be identified in the highly congested ribityl region of the spectrum (Figure 5).[24] Few changes were observed in the chemical shifts of the C4′ and C5′ signals of the ribityl side chain.

Integration of the five assigned signals for each carbon atom allowed one to subdivide the spectrum into five sets of 13 lines, each of whose carbon assignment is known (Table III). The five anionic lumazines are proposed as the 7α exo methylene compound, diastereomeric five-membered ring ethers, and diastereomeric six-membered ring ethers (Figure 2). The formation of six-membered ring ethers was supported by noting the downfield shifts of the C3′ resonance (δ 77.5 and 79.5 compared to 76.4 in the neutral lumazine). The carbon signals of C2′ of the six-membered ring ethers appeared upfield from the neutral species. The same characteristic downfield shifts of the α carbon and upfield shifts of the β carbon are observed

(24) See paragraph at end of paper about supplementary material.
(25) Abbreviation: UV, ultraviolet; NMR, nuclear magnetic resonance; TSP, 3-(trimethyl)propionic acid, sodium salt; Me_2SO, dimethyl sulfoxide; 2D NMR, two-dimensional NMR; NOE, nuclear Overhauser effect.

Table IV. ^{1}H NMR Chemical Shifts of 6,7-Dimethyl-(D-ribityl)lumazine[a]

			anionic compounds			
			five-membered rings		six-membered rings	
proton assignment	neutral compound	7α-exo methylene	major	minor	major	minor
6α	2.69	2.15	2.19	2.10	2.12	2.16
7α	2.90	4.34, 4.39	1.38	1.30	1.52	1.58

[a] Proton assignments confirmed by 2D NMR heteronuclear chemical shift correlation experiment.

in the five-membered ring ether anions. Only minor changes were observed in the chemical shifts of C4′ (δ 75) and C5′ (δ 65), suggesting no seven-membered ring ether formation.

^{1}H NMR of 6,7-Dimethyl-8-(D-ribityl)lumazine. The relevant spectral data of 6,7-dimethyl-8-(D-ribityl)lumazine recorded at pH 7 are given in Table IV. The ^{1}H resonances of 6α (δ 2.69) and 7α (δ 2.90) are easily assigned due to deuterium exchange at the acidic 7α position. The 7α protons consist of a singlet for the CH_3 species accompanied by a 3-line pattern (J = 2 Hz) which is shifted 0.02-ppm upfield due to the two-bond deuterium isotope effect of the CH_2D species. The 7α protons are completely exchanged by recording ^{1}H NMR spectra in D_2O buffered solutions. The assignments of the ribityl protons were determined by homonuclear selective decoupling experiments. The observed coupling constants suggest an average conformation as shown in Figure 2 with an extended ribityl side chain.

The ^{1}H NMR of the 8-(D-ribityl) compound at pH 11 confirmed the existence of five major anionic species (Table IV). The peaks of the neutral species (δ 2.69, 6α and δ 2.90, 7α) are not observed on the NMR time scale. All five of the 6α methyl singlets are resolved and resonate at δ 2.10–2.18. Only four 7α signals with upfield deuterium isotope satellites due to CH_2D species are present at δ 1.30–1.58. The upfield signals (δ 1.30 and 1.38) are of the five-membered ring ether anions and the downfield pair (δ 1.52 and 1.58) are assigned to the 7α protons of the six-membered ring ether diastereomers. The 7α signals for the fifth species are the multiplets at δ 4.34 and 4.39. These signals integrate for two protons and are assigned to the olefinic 7α protons of the exo methylene anion. All the 7α methyl and the majority (≥80%) of the exo methylene protons have undergone exchange when the ^{1}H NMR spectrum is measured in D_2O 5 min after dissolving the lumazine. Integration of the 6α and 7α ^{1}H resonances of the five major anionic lumazines gave the same equilibrium ratio as calculated from ^{13}C NMR integrations (Table II and Figures 3 and 4).[24] ^{1}H-^{13}C correlations in the crowded ribityl proton regions were also observed by 2D NMR but were not completely interpretable.

Lumazines with One Anionic Species. Three 6,7-dimethyl-8-substituted-lumazines were determined to exist only as the 7α exo methylene anionic compound in basic solution. These were the 8-methyl, 8-(5-hydroxypentyl), and 8-(6-hydroxyhexyl) derivatives which also have comparable pK_a values for the 7α protons (Table II). Analysis of the ^{13}C NMR spectral data (Table VIII)[24] from 6,7,8-trimethyllumazine allows straightforward assignments because intramolecular ether formation cannot occur. The 8-(6-hydroxyhexyl) and 8-(5-hydroxypentyl) analogues also showed no evidence of intramolecular ethers. The formation of eight- and nine-membered ring ether anionic lumazines is not observed at equilibrium in alkaline solution. The ^{13}C NMR data (Table VIII)[24] show characteristic peaks at δ 24 (C6α), δ 89 (C7α), δ 107 (C4a), δ 142 (C7), and δ 161 (C2) for the 7α exo methylene anionic compounds. The ^{1}H NMR data (Table IX I, J, K)[24] are also consistent with only one anionic lumazine at pH 11.

Lumazines with Two Anionic Species. Three additional 8-(ω-hydroxyalkyl)-6,7-dimethyllumazines were studied to provide additional evidence for intramolecular ether formation. The 8-(2-hydroxyethyl) analogue showed the formation of both the 7α exo methylene (59%) and five-membered ring ether (41%) anions. Likewise, the 8-(3-hydroxypropyl) compound was determined to exist as predominantly (89%) the 7α exo methylene anion with only 11% intramolecular six-membered ring ether formation. The cyclization of the 8-(4-hydroxybutyl) analogue forming a seven-membered ring was unexpected since only five- and six-membered rings were observed for the D-ribityl compound.

The ^{13}C NMR (Table VII) and ^{1}H NMR (Table IX F, G, H)[24] data for the 7α exo methylene species are similar to those previously discussed. Characteristic chemical shifts for carbons 2, 4a, 6α, 7, and 7α and the 6-methyl (δ 2.10) and 7-methylene protons (δ 4.25 and 4.35) were observed. In the cyclic ethers C2 resonates at δ 162, C4a at δ 103–105, C6α at δ 21, C7 at δ 91.5–93, and C7α at diagnostic chemical shifts depending at the ring size. The most upfield resonance at δ 18 is comparable to the C7α signal of the minor six-membered ring diastereomer of the 8-(D-ribityl) anionic lumazines. The ^{13}C NMR chemical shift of C7α of the five-membered ring ether anion from the 8-(2-hydroxyethyl) analogue (δ 23) is similar to the major diastereomer of the D-ribityl compound (Table III).

The ^{1}H NMR chemical shifts of 7α for the five- and six- and seven-membered ring ether anions are also distinctive. The seven-membered ring anionic compound has a H7α resonance at δ 1.42 (Table IX, H).[24] There is no comparable signal and consequently no seven-membered ring formation in the D-ribityl compound (Table IV). The six-membered ring ^{1}H chemical shift of H7α (δ 1.54, Table IX, G)[24] substantiates our claim of six-membered ring ether formation in the natural occurring lumazine (δ 1.52 and 1.58, Table IV). The chemical shift of H7α (δ 1.30) of the five-membered ring ether anion is also diagnostic. Since the (ω-hydroxyalkyl)lumazine cyclic ether anions have only one chiral center (at C7), enantiomers are formed which are indistinguishable by normal NMR techniques.

Lumazines with Three Anionic Species. The formation of diastereomeric intramolecular ether anions was investigated in the cases of 8-(D,L-2-hydroxypropyl)- and 8-(2-deoxy-D-ribityl)-6,7-dimethyllumazine. Five-membered ring ether formation was highly favored (45% and 39%) in the 8-(D,L-2-hydroxypropyl) analogue. This is also reflected in the considerably lower value for the pK_a of the 7α protons (8.90, Table II). Only diastereomeric six-membered ring ethers (16% and 5%) were observed for the 8-(2-deoxy-D-ribityl) compound lacking a 2′-hydroxyl group. There is no evidence for the formation of diastereomeric seven-membered ring anionic species. Beach and Plaut[8] had previously reported the ^{1}H NMR of the 8-(2-deoxy-D-ribityl) as well as the 8-(3-hydroxypropyl) analogue and concluded that no intramolecular six-membered ring ethers were formed.

Lumazines with Five Anionic Species. In addition to the D-ribityl compound, the D,L-glyceryl-, D-arabityl-, and D-xylityllumazines were analyzed. The observation

of five species at equilibrium in alkaline solution of 6,7-dimethyl-8-(D,L-glyceryl)lumazine is significant since no large rings are possible. The five- or six-membered ring ether carbons at C2′ (CH) and C3′ (CH_2) were easily distinguished by a ^{13}C NMR experiment using gated ^1H decoupling. The characteristic upfield shift of C2′ and downfield shift of C3′ was observed in the formation of the six-membered rings (Table V, B).[24] The ^1H NMR (Table IX, D)[24] chemical shifts of five-vs. six-membered ring methyls at 6α (δ 2.16 and 2.14 vs. δ 2.09 and 2.04) and 7α (δ 1.36 and 1.30 vs. δ 1.52 and 1.50) support the equilibrium ratios given in Table II.

Slightly more (41% and 27%, Table II) intramolecular cyclization of the 3′-hydroxyl was observed in the cases of the 8-(D-arabityl)- and 8-(D-xylityl)-6,7-dimethyllumazines. Further interpretation of these results is hampered by our present inability to assign unambiguously the configurations of the diastereomers. Consequently, the equilibrium diastereomeric ratios for the cyclic ether anions listed in Table II are arbitrary and could be reversed. We attempted one- and two-dimensional NOE measurements in H_2O, because of the exchange of the 7α methyl group in D_2O, but observed no NOE between the H2′ and/or H3′ signals and the 7-CH_3. It should be emphasized that the assignments of the five- (H7α at δ 1.30 to 1.39) and six-membered ring (δ 1.5) ether anions is not arbitrary. The ^1H NMR data for 8-(D-xylityl)- and 8-(D-arabityl)-6,7-dimethyllumazines (Table IX, D, C)[24] revealed the equilibrium ratios given in Table II. The pK_a values of the three 8-pentityl analogues are the same (8.3) even though there are minor differences in the equilibrium compositions.

Conclusions. It is clear that the addition of a substituent on the terminal end of the side chain increases the formation of cyclic ether anions. For example, 41% of the 2′-hydroxyethyl compound exists as the five-membered ring ether. This is more than doubled to 84% by the addition of a methyl group at C2′ in the 8-(D,L-2-hydroxypropyl) analogue. Similarly, going from 3′-hydroxypropyl to 2-deoxy-D-ribityl increases the formation of six-membered rings from 11% to 21%. In the case of 6,7-dimethyl-8-(D,L-glyceryl)lumazine only 12% of diastereomeric six-membered ring ether anions were observed (Table II). However, in the tetrahydroxypentyl derivatives (ribityl, arabityl, and xylityl) the equilibrium amount of six-membered ring ether anions increases from 21% to 41%.

The formation of the seven-membered ring ether anion of the 8-(4-hydroxybutyl) analogue deserves additional comment. The seemingly greater ease of formation of six vs. seven-membered ring intramolecular ethers in the polyhydroxypentyl derivatives is viewed in terms of nonbonding interactions of the solvated hydroxyl groups. Considerably less solvation by water molecules is expected in the ω-hydroxyalkyl series in which the seven-member ring formation is favored over the six-membered ring. It should be noted that the five-membered ring ether of lumazines bearing a 2′-hydroxyl group are the most stable ethers in each series. The five-, six-, and seven-membered ring ether and exo methylene anions listed in Table II are energetically similar (< 1 kcal/mol difference) as seen by the equilibrium ratios. Consequently, molecular model studies of the various species in solution did not prove useful in rationalizing the minor differences in thermodynamic stabilities. We have no ^1H or ^{13}C NMR evidence in neutral or basic solution for the hydration by a molecule of water of any of the lumazines of this study. We cannot rule out the possibility of such species occurring transiently, but they represent no significant (i.e., observable by NMR) fraction of the equilibrium mixture.

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Registry No. 6,7-Dimethyl-8-D-ribitylumazine, 2535-20-8; 6,7-dimethyl-D-arabityllumazine, 53176-76-4; 6,7-dimethyl-8-D-xylityllumazine, 5022-75-3; 6,7-dimethyl-8-DL-glyceryllumazine, 31790-90-6; 6,7-dimethyl-8-(DL-2′-hydroxypropyl)lumazine, 102285-17-6; 6,7-dimethyl-8-(2′-hydroxyethyl)lumazine, 13300-17-9; 6,7-dimethyl-8-(2′-deoxyribityl)lumazine, 31735-32-7; 6,7-dimethyl-8-(3′-hydroxypropyl)lumazine, 31735-34-9; 6,7-dimethyl-8-(4′-hydroxybutyl)lumazine, 102285-18-7; 6,7-dimethyl-8-(5′-hydroxypentyl)lumazine, 102285-19-8; 6,7-dimethyl-8-(6′-hydroxyhexyl)lumazine, 102285-20-1; 6,7-dimethyl-8-methyllumazine, 5784-00-9; 6-[(4′-hydroxybutyl)-amino]-5-nitroso-2,4(1*H*,3*H*)-pyrimidinedione, 102285-21-2; 4-amino-1-butanol, 13325-10-5; 6-chloro-2,4(1*H*,3*H*)-pyrimidinedione, 4270-27-3; 5-amino-1-pentanol, 2508-29-4; 6-[(5′-hydroxypentyl)amino]-5-nitroso-2,4(1*H*,3*H*)-pyrimidinedione, 102285-22-3; 6-[(6′-hydroxyhexyl)amino]-5-nitroso-2,4(1*H*,3*H*)-pyrimidinedione, 102285-23-4; DL-1-amino-2-propanol, 1674-56-2; 6-[(DL-2′-hydroxypropyl)amino]-5-nitroso-2,4(1*H*,3*H*)-pyrimidinedione, 102285-24-5; diacetyl, 431-03-8; 6-[(4′-hydroxybutyl)amino]-5-amino-2,4(1*H*,3*H*)-pyrimidinedione, 102285-25-6; 6-[(5′-hydroxypentyl)amino]-5-amino-2,4(1*H*,3*H*)-pyrimidinedione, 102285-26-7; 6-[(6′-hydroxyhexyl)amino]-5-amino-2,4(1*H*,3*H*)-pyrimidinedione, 102285-27-8; 6-[(DL-2′-hydroxypropyl)-amino]-5-amino-2,4(1*H*,3*H*)-pyrimidinedione, 102285-28-9.

Supplementary Material Available: Tables V–IX and Figures 4 and 5, giving ^{13}C and ^1H NMR data on a number of 6,7-dimethyl-8-substituted-lumazines (10 pages). Ordering information is given on any current masthead page.