171. The Structure of Covalent Hydrates[1]) of Alloxazines[2])

A Reinvestigation

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Summary

It is shown by ^{13}C-NMR. and synthesis of 6,7-dimethyl-3-ethoxycarbonylamino-quinoxaline-2-carboxamide (**5**) and methyl 6,7-dimethyl-3-(*N'*-methylureido)-quinoxaline-2-carboxylate (**6**) that hydrolysis of lumichrome (**1**, R = H) must yield 6,7-dimethyl-3-ureidoquinoxaline-2-carboxylic acid (**2**, R = H) and not 6,7-dimethyl-2-oxo-1,2,9,9a-tetrahydrooxazolo[4,5-*b*]quinoxaline-9a-carboxamide (**2a**, R = H), as previously proposed.

Introduction. – As mentioned in the literature [1] [2], alloxazines unsubstituted at N(1) undergo a reversible reaction in aqueous alkaline solution yielding 'covalent hydrates'. For the product obtained from lumichrome[4]) the structure **2a** (R = H) *(cf. Scheme)* has been proposed [3]. More recent data [4], however, seem inconsistent with the proposed structure. Therefore, the structure of the 'covalent hydrates' was reinvestigated using ^{13}C-NMR. and chemical synthesis. We show that the correct structure of the 'covalent hydrate' of alloxazine is 6,7-dimethyl-3-(*N'*-methylureido)quinoxaline-2-carboxylic acid (**2**, R = CH_3).

Results and discussion. – For the unambiguous synthesis of the 'covalent hydrate' two routes were chosen, with methyl 3-amino-6,7-dimethyl-quinoxaline-2-carboxylate (**3**) *(cf. Scheme)* as the starting material. The ester **3** was used because the corresponding free acid undergoes easy decarboxylation under the conditions of the synthesis. The free acid corresponding to **3** is, in fact, a product of exhaustive hydrolysis of lumichromes (**1**, R = H or CH_3) [8]. The aim of the synthesis of **5** and **6** was to compare the physical and chemical properties of these compounds

[1]) For the term 'covalent hydrates', see [1].
[2]) Alloxazine = 1*H*,3*H*-benzo[g]pteridine-2,4-dione.
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[4]) Lumichrome = 7,8-dimethylalloxazine.

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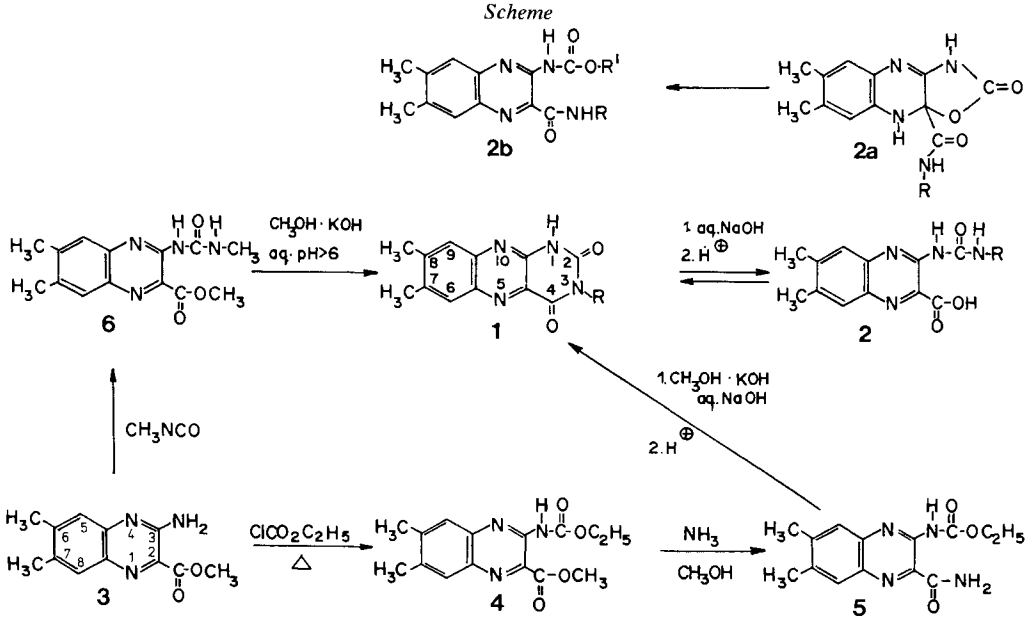
Scheme



with those of the esters of the 'covalent hydrates' of **1** for which structure **2b** was proposed. Compound **5** but not **6** should be identical with **2b** ($R = H$, $R' = C_2H_5$).

The syntheses of **5** and **6** were similar to the procedures described by *Clerin et al.* [12] for the preparation of isoalloxazine (= flavin).

a) A solution of **3** in CH_3NCO yields **6** in a slow reaction and also some **1** ($R = CH_3$). Compound **6** possesses the same physical and chemical properties *(cf. Table 1)* as the methyl ester of **2** ($R = CH_3$) and can be converted to **1** ($R = CH_3$) in aqueous solutions of pH > 6 or in methanolic KOH-solution.

b) The second synthesis consisted of the transformation of **3** into **4** by ethyl chloroformate. The product **4** was in turn converted to the amide **5** which is different from the ethyl ester of the 'covalent hydrate' [3] with $R = H$ (see *Table 1*). The conditions for the conversion of **5** into **1** ($R = H$) are less mild than those for the conversion of **6** → **1** ($R = CH_3$).

From these results it follows that **2** is the true structure of the 'covalent hydrate'.

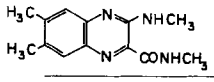
This structure was confirmed by ^{13}C-NMR. measurement of selectively ^{13}C-enriched compounds. The most significant difference between **2** and **2a** is the state of hybridization of the C(4a)-atom (after numbering of flavins). Therefore, the chemical shift of C(4a) provides independent and unequivocal evidence for the real structure. The results are collected in *Table 2*. The chemical shifts are in agreement with structure **2** for the 'covalent hydrate' but not with the earlier proposed structure **2a**. In the latter case the resonance signal of C(4a) would be expected at much higher field, similar to that of a C(4a)-substituted flavin [13]. The normalized intensities of the signals of proton noise decoupled spectra *(Table 2)* also agree with structure **2** ($R = CH_3$), but not with structure **2a** ($R = CH_3$).

Table 1. *Comparison of some physical data of* **5**, **6** *and methyl and ethyl ester of* **2**

Technique	Compound			
	6	**2** (methyl ester) ($R = CH_3$)[a]	**5**	**2** (ethyl ester) ($R = H$)[b]
UV. (methanol)	376 nm (3.70)	376 nm (3.70)	365 nm (3.83)	378 nm (3.76)
λ_{max} ($\log\varepsilon$)	334 nm (3.78)	334 nm (3.78)	320 nm (3.80)	318 nm (3.83)
IR. $\tilde{\nu}$(NH) [cm^{-1}]	3320, 3270	3320, 3260	3460, 3260	3310, 3180
IR. $\tilde{\nu}$(CO) [cm^{-1}]	1687	1690	1740, 1675	1725, 1695
^{1}H-NMR.[c]	2.44 ($CH_3C(7)$);	2.44 ($CH_3C(7)$);	1.5–1.3 (CH_3)[f];	1.55–1.40 (CH_3)[f];
	2.50 ($CH_3C(6)$);	2.52 ($CH_3C(6)$);	2.45 ($CH_3C(7)$);	2.40 ($CH_3C(7)$);
$CDCl_3$	3.07–2.98[d]) ($CH_3N(3)$);	3.10–3.00 ($CH_3N(3)$);	2.55 ($CH_3C(6)$);	2.45 ($CH_3C(6)$);
	4.05 (CH_3O);	4.05 (CH_3O);	4.6–4.2 (CH_2)[g];	4.65–4.45 (CH_2)[g];
	7.52 (H–C(5));	7.57 (H–C(5));	7.5 (NH_2);	7.55 (H–C(5));
	7.81 (H–C(8));	7.83 (H–C(8));	7.85 (H–C(5));	7.85 (H–C(8));
	9.3 (H–N(3))[e]);	9.21 (H–N(3))[e]);	8.00 (H–C(8));	10.28 (H–N(3)
	10.12 (H–N′(3))[e])	10.10 (H–N′(3))[e])	8.40 (H–N(3) and H–N′(3))[e])	and H–N′(3))[e])

[a]) Published data [3] for the proposed structure **2b** ($R = R' = CH_3$). [b]) According to [3] this compound possesses structure **2b** ($R = H$, $R' = C_2H_5$). [c]) Ring numbering according to alloxazine system *(cf. Scheme)*. [d]) *d*, $J = 5$ Hz, proton exchangeable with deuteron (3.03 ppm). [e]) *m*, proton exchangeable with deuteron. [f]) *t*. [g]) *qa*.

Table 2. *The relevant ^{13}C-chemical shifts* (in ppm) *and the normalized intensities of the corresponding resonance lines of alloxazines and their 'covalent hydrates'*

Compound	Solvent	Chemical Shifts (Intensities)			
		C(2)	C(4)	C(4a)	C(10a)
1 (R = CH_3)[a]	DMSO	150.35 (101)	160.61[b] (20) 160.32[b] (27)	129.53 (34)	145.39[b] (37) 145.10[b] (30)
2 (R = CH_3)[a][d]	DMSO	154.22 (140)	167.34[c] (25) 167.14[c] (30)	131.28 (45)	146.73[c] (30) 146.56[c] (35)
6 [d][e]	$CDCl_3$	154.71 (120)	165.48 (45)	135.63 (50)	146.53 (100)
 [d][e][f]	$CDCl_3$	–	166.39 (100)	133.03 (60)	152.37 (115)

[a]) Compounds selectively ^{13}C-enriched, s. experimental part. [b]) *d*, $^2J_{C(4),C(10a)} = 7.2$ Hz. [c]) *d*, $^2J_{C(4),C(10a)} = 5.0$ Hz. [d]) Ring numbering is according to that of alloxazine. [e]) Natural abundance spectra. [f]) Prepared as described in [14].

Structure **2** is also in agreement with the published physical and chemical data for the 'covalent hydrate' [3] which, however, have led to the proposal of structure **2a** by an incorrect interpretation.

Other observations can now be explained. Thus, N(1)-substituted and N(1),N(3)-unsubstituted alloxazines are very stable towards hydrolysis at high pH values (*ca.* 14), in contrast to N(1),N(3)-dialkylated compounds which hydrolyze easily to *N*-alkyl-3-alkylamino-6,7-dimethyl-quinoxaline-2-carboxamide [14]. This means that whenever the N(3)-position is ionized the carbonyl functions at positions 2 and 4 are protected from attack by hydroxyl ions. Only at pH < p*K* of N(3) (*ca.* 12.5 for the dianion and lower for the monoanion) can hydrolysis occur.

On the other hand, N(3)-alkylated alloxazines form the N(1) anion with a p*K* of 8.50 [5]. The negative charge of this species, mainly localized on the N(1)–C(2)=O region of the molecule, protects C(2)=O but allows attack of C(4)=O by hydroxyl ions and at the same time protects the product from further hydrolysis.

The reversible formation of **1** from **2** is surprising, although precedence for intramolecular nucleophilic attack of an ureido group upon a carboxyl group exists: *Hegarty & Bruice* [15] found that 2-ureido-benzoic acid undergoes a cyclization which is irreversible in weak acid or alkaline aqueous solution. In addition *Smith & Bruice* [16] have reported that alkaline hydrolysis of isoalloxazines (flavins) yields the corresponding, but intermediate product of **2** which reacts reversibly under certain conditions. Therefore it must be concluded that hydrolysis of alloxazines and isoalloxazines follow the same route with respect to the initially formed product.

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Experimental Part

General. UV./VIS. spectra were recorded on a *Cary* 118c spectrophotometer. IR.-spectra (KBr pellets) were measured on a *Specord* IR.-75 spectrophotometer. The ^{1}H-NMR. spectra were taken on a 60 MHz *Varian* 360 A spectrometer. The ^{13}C-NMR. spectra were recorded either on a *JEOL* FX-60 or on a *Varian* XL-100 spectrometer operating at 25.2 MHz. All ^{13}C-NMR. spectra were acquired in the *Fourier* transform mode using 12 mm taperlock tubes and under proton noise decoupling conditions. TMS was used as an internal standard. Mass spectra were obtained on a *JEOL* D-100 spectrometer (*m/z* values were given).

Melting points (m.p.) are uncorrected. The purity of the compounds was judged by thin layer chromatography (TLC.) (silica gel 1B2, *Bakerflex*) with $CHCl_3$/2-butanone 14:1 (*v/v*).

Lumichrome (**1**, R = H) and *N(3)-methyllumichrome* (**1**, R = CH_3) were prepared according to published procedures [5] [6]. The synthesis of the 'covalent hydrate' of **1** (R = H, CH_3) has been described [3]. Compound **3** was prepared by methylation of 3-amino-6,7-dimethylquinoxaline-2-carboxylic acid [7] obtained according to the procedure of *McNutt* [8].

^{13}C-labelled N(3)-methyllumichrome ($^{13}C_4$-**1**, R = CH_3). An equimolar mixture (70 mg) of [4,10a-$^{13}C_2$]-, [4a-^{13}C]- and [2-^{13}C]-*N*(3)-methyllumiflavin [9] was dissolved in 1 ml DMF in a test tube, 3 g of urea was added and the mixture heated at 120° for 24 h. After this time the reaction was almost completed (TLC.). The gel-like content of the test tube was then cooled to RT. and added to 10 ml 2M aqueous acetic acid. The suspension was extracted with $CHCl_3$, the organic phase thoroughly washed with water and dried (Na_2SO_4). The $CHCl_3$ phase was evaporated, the residue dissolved in a mixture of $CHCl_3$/2-butanone 14:1 (*v/v*) and chromatographed through a short column [10]. The fraction containing $^{13}C_4$-**1** was evaporated to dryness and the residue crystallized from methanol yielding 41 mg (62%) of pure compound, m.p. 225° ([5]: 225°).

The 'covalent hydrate' of $^{13}C_4$-**1** was prepared as described in [3].

6,7-Dimethyl-3-ethoxycarbonylaminoquinoxaline-2-carboxamide (**5**). Dry NH_3 was slowly purged into a solution of 100 mg of **4**, obtained from **3** [11], in 40 ml of methanol at 24° until **4** no longer was detectable by TLC. The solution was then rapidly evaporated i.v. to dryness at < 40°. The crude product thus obtained was twice crystallized from methanol giving 62 mg (65%) of pure **5**, yellow needles, m.p. 235°. – MS.: 288 (M^+). The compound possessed a green fluorescence. For other physical data, see *Table 1*.

Methyl 6,7-dimethyl-3-(N'-methylureido)quinoxaline-2-carboxylate (**6**). A solution of 40 mg of **3** in 20 ml of freshly distilled methyl isocyanate was kept in the dark at RT. for about 72 h. Some **1** (R = CH_3) was formed besides **6** (TLC.). The reaction mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ from which **6** was obtained pure (17 mg, 34%) as pale yellow crystals by the addition of isopropyl ether; m.p. 186°. – MS. (*m/z*): 288 (M^+). For other physical data, see *Table 1*.

Ethyl ester of 'covalent hydrate' was prepared from the 'covalent hydrate' of **1** (R = H) following the procedure for the methyl ester [3]. The crude product was dissolved in a mixture of $CHCl_3$/2-butanone 14:1 (*v/v*) and chromatographed [10]. The main fraction was collected, evaporated to dryness and the residue crystallized from ethanol as pale yellow crystals (66%); m.p. 224°. – MS.: 288 (M^+). For other physical data see *Table 1*.

Synthesis of lumichrome (**1**, R = H) *and N(3)-methyllumichrome* (**1**, R = CH_3). – a) *From* **5**. To a solution of 100 mg of **5** in 18 ml of methanol was added 2 ml of 1M methanolic KOH-solution. The reaction is completed in a few hours at RT. (TLC.). The reaction mixture was neutralized with 1M HCl, the precipitate filtered off and crystallized from methanol. The yield of **1** (R = H) was 70 mg (83%).

b) *From* **6**. To a solution of 100 mg of **6** in 18 ml of methanol was added 2 ml 1M methanolic KOH-solution. The reaction is complete within a few minutes. The precipitate, obtained after neutralization of the reaction mixture with 1M HCl, was collected and crystallized from methanol. The yield of **1** (R = CH_3) was 75 mg (84%).

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