300. Natural Abundance ^{13}C-NMR. of Reduced Isoalloxazine[1])

Preliminary Communication

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Summary

When an isoalloxazine derivative (**1**) was reduced (**2**) its ^{13}C-NMR. resonances were all shifted upfield and the largest shifts were observed for C(4a), C(6) and C(8). In contrast, the spectrum of a model for 4a,5-dihydro-isoalloxazine (**3**) showed upfield shifts for C(7) and C(8) but downfield shifts for C(2), C(4) and C(10a). The results and their biochemical implications are shortly discussed.

We have previously reported on the ^{1}H- and ^{13}C-NMR. characteristics of isoalloxazines in the oxidized state [1] [2]. In contrast to the oxidized molecule the two-electron reduced state has not received the same detailed attention because of its easy oxidation by molecular oxygen. The chemical properties of reduced isoalloxazine are still not well understood. This fact prompted us to investigate the reduced molecule by ^{13}C-NMR. technique. Since this method yields information on the electron distribution in a molecule it is expected that such an investigation will contribute to a better understanding of the chemical properties of reduced isoalloxazine. In addition our current NMR. studies on flavoproteins made it necessary to characterize the free, reduced isoalloxazine by ^{13}C-NMR.

The structures of the compounds investigated in this study are given in the *Scheme*. Two different forms of reduced isoalloxazine have been studied, namely the 1,5-dihydro form **2** and the 4a-benzyl-4a,5-dihydro form **3**. It is believed [3] that of all dihydro derivatives known these two are the most common in biology. Compound **3** was synthesized according to known procedures [4]. Compound **2** was obtained from **1** and was generated *in situ* in the 12 mm NMR. tube by the following procedure. A 50 mM solution of **1** in $CDCl_3$ (2 ml) was placed in the NMR. tube and 2 ml of an aqueous (phosphate buffer, 0.5 M, pH 8.0) solution saturated with NaCl was added. The aqueous phase contained at least a 10fold molar excess

[1]) Isoalloxazine = 10-substituted 2,3,4,10-tetrahydrobenzo[*g*]-pteridine-2,4-dione; flavin = 7,8-dimethyl-isoalloxazine; lumiflavin = 7,8,10-trimethyl-isoalloxazine.

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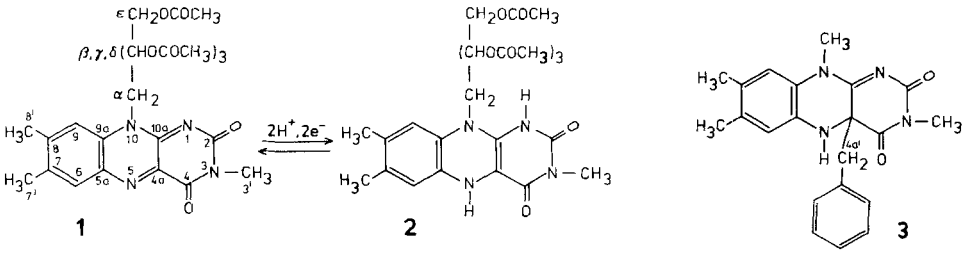
Scheme



of $Na_2S_2O_4$ as reducing agent to prevent reoxidation of **2** during the experiments. Prior to the addition of $Na_2S_2O_4$ the aqueous solution was flushed with N_2 to remove most of the O_2. The NMR. tube was made gastight by means of a taperlock. It was then shaken vigorously to obtain **2** which could be recognized by its orange colour and by its lack of fluorescence. A 50 mM solution of **3** in $CDCl_3$ is stable provided that light or oxygen are excluded.

The ^{13}C-NMR. spectra were recorded on a *Varian* XL-100 high resolution spectrometer operating at 25.2 MHz and equipped with a 16 K *Varian* 620-L computer. All spectra were acquired in the *Fourier* transform mode. Internal deuterium served as a frequency lock signal. TMS was used as internal standard. Typical instrumental conditions were: pulsewidth 10 μs (30° pulse), repetition rate 2s, spectral width 5000 Hz, number of transients *ca.* 10.000.

The natural abundance spectra of **1** and **2** are shown in the *Figure* and the results obtained from **1**, **2** and **3** are collected in the *Table*. The assignment of the quaternary carbon atoms in position 2,4,4a and 10a of the isoalloxazine ring was made by using compounds selectively enriched with ^{13}C [2]. The other carbon atoms were assigned according to results obtained by various NMR. techniques (proton

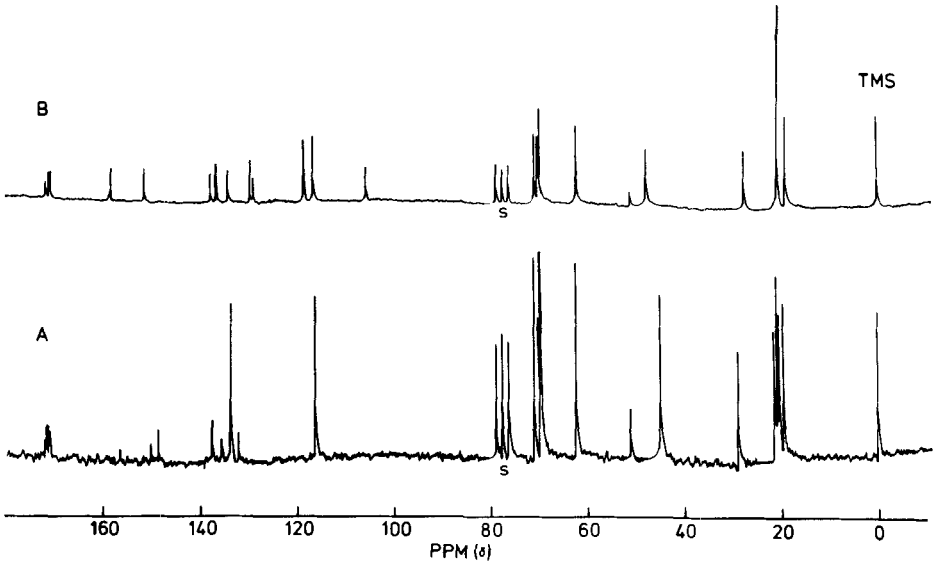


Figure. *Natural abundance proton noise decoupled* ^{13}C*-NMR. spectra of* **1** (A) *and* **2** (B) *in* $CDCl_3$ (s = resonances due to the solvent)

Table. ^{13}C *Chemical shifts* δ (in ppm relative to TMS) *of* N(3)*-methyl-tetraacetylriboflavin in the oxidized* (**1**) *and the 1,5-dihydro state* (**2**) *and of* N(3)*-methyl-4a-benzyl-4a,5-dihydro-lumiflavin* (**3**) *in* $CDCl_3$

Carbon atom	Chemical shifts in compound				
	1	**2**	$\Delta\delta$[a])	**3**	$\Delta\delta$[b])
C(2)	155.9	150.6	5.3	161.7	−11.1
C(4)	161.4	157.7	3.7	169.1	−11.4
C(4a)	136.0	105.2	30.8	59.0	46.2
C(5a)	134.9	136.0	−1.1	-[c])	-
C(6)	133.1	116.1	17.0	117.3	−1.2
C(7)	136.8	133.6	3.2	110.4	23.2
C(8)	147.7	129.0	18.7	110.0	19.0
C(9)	115.6	118.0	−2.4	117.5	0.5
C(9a)	131.9	128.2	3.7	-[c])	-
C(10a)	149.2	137.1	12.1	155.6	−18.5
C(7′)	19.4	18.9	0.5	19.4	−0.5
C(8′)	21.4	18.9	2.5	19.4	−0.5
C(3′)	28.7	27.3	1.4	27.7	−0.4
C(10′α)	45.3	47.4	−2.1	-	
C(10′)	-	-		32.1	
C(4a′)				43.5	
C(10′ε)	62.0	62.0			
C(10′β,γ,δ)	69.2	69.7			
	69.5	70.0			
	70.6	70.1			
CH_3CO(10′$\beta,\gamma,\delta,\varepsilon$)	21.0	20.6			
	20.8	20.6			
	20.7	20.6			
	20.3	20.6			
CH_3CO(10′$\beta,\gamma,\delta,\varepsilon$)	170.8	170.8			
	170.5	170.1			
	170.1	170.0			
	169.9	169.8			

[a]) Difference in chemical shifts between **1** and **2**, negative values are to low field.
[b]) Difference in chemical shifts between **2** and **3**, negative values are to low field.
[c]) Could not be observed.

noise decoupling, off-resonance and gated decoupling techniques) and by the use of derivatives with varying degrees of methyl substitution in the benzene subnucleus of the isoalloxazine molecule [1].

Some of the resonances due to the side chain of **1** and **2** could not be assigned to individual carbon atoms because of lack of resolution *(Table)*. Nevertheless it can be seen that the resonances of the methyl groups of the acetyl groups become magnetically equivalent upon reduction of **1**, indicating a small (conformational) perturbation of the side chain when going from **1** to **2**.

A comparison of the difference of chemical shifts of the carbon atoms of the isoalloxazine molecule *(Table)* reveals that, with the exception of the three carbon atoms C(5a), C(9) and C(10′α), the carbon atoms in **2** resonate at higher fields than those of **1**. This observation is in accord with expectation when an electron-deficient compound is transformed into an electron-rich one. However, there are some remarkable differences with respect to the upfield shift of resonances of particular

carbon atoms. The largest upfield shift is observed for the resonance due to C(4a). The upfield shift of 30.8 ppm indicates a considerable increase in π-electron density at C(4a) in **2** as compared to **1**. Upfield shifts of 17.1, 18.7 and 12.1 ppm are observed for C(6), C(8) and C(10a), respectively. In a previous paper [2] it was suggested that these carbon atoms are positively charged in **1** due to rather strong conjugation with the carbonyl group at position 2. It is known from crystallographic studies [5] that reduced isoalloxazines are bent. Such a bent conformation should reduce the influence of the carbonyl at position 2 on the C(6), C(8) and C(10a) atoms, a hypothesis which seems to be supported by the present results. On the other hand, the downfield shift of the resonances due to C(9), C(5a) and C(10'a), and the fact that C(10a) is less affected than C(6) and C(8) when **1** is reduced, suggests that in **2** some conjugation through N(1) to C(2) exists but it involves C(9) and C(5a) and occurs through N(10) and C(10a). To make such a conjugation possible the molecule would have to assume a more planar conformation involving the atoms mentioned above. If this idea is correct it might be a clue to the different reactivities of reduced protein-bound isoalloxazine towards molecular oxygen, the reactivity being determined by the particular conformation of reduced isoalloxazine imposed on it by the interaction with the apo-protein. A detailed study to pursue this idea in more detail is currently being conducted.

Compound **3** has been choosen as a model for the proposed interaction of reduced isoalloxazine with molecular oxygen yielding a C(4a)-hydroperoxide [6]. In compound **3** the resonance most affected is that due to the C(4a) atom where the alkyl group is attached. The further upfield shift of 46.2 ppm as compared to **2** is in accord with a change of hybridization ($sp^2 \rightarrow sp^3$) of the C(4a) atom. As compared to **2** the resonances due to C(2), C(4) and C(10a) in **3** are less shielded than those in **1**. Furthermore the C(7'), C(8') and C(7), C(8) atoms in **2** and the C(7'), C(8') atoms in **3** are magnetically equivalent whereas these atoms in **1** are magnetically non-equivalent. These results indicate that the ^{13}C-NMR. spectrum of a C(4a)-hydroperoxide intermediate, if it can be trapped, should exhibit a chemical shift in the range of about 80–60 ppm for the C(4a) atom.

A detailed paper on this subject will be published in this journal.

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REFERENCES

[1] *H.J. Grande, C.G. van Schagen, T. Jarbandhan & F. Müller*, Helv. *60*, 348 (1977).
[2] *H.J. Grande, R. Gast, C.G. van Schagen, W.J.H. van Berkel & F. Müller*, Helv. *60*, 367 (1977).
[3] 'Flavins and Flavoproteins', *T.P. Singer*, ed., Elsevier Publishing Company, Amsterdam 1976.
[4] *W.H. Walker, P. Hemmerich & V. Massey*, Helv. *50*, 2269 (1967).
[5] *P. Kierkegaard, R. Norrestam, P.E. Werner, I. Csöregh, M. van Glehn, R. Karlsson, M. Leijonmarck, O. Rönquist, B. Stensland, O. Tillberg & L. Torbjörnsson* in 'Flavins and Flavoproteins', H. Kamin, ed., University Park Press, Baltimore 1971, p. 1.
[6] *S. Ghisla, B. Entsch, V. Massey & M. Husein*, European J. Biochemistry *76*, 139 (1977).