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On the ^{1}H NMR Spectra of Biliverdins with Free Propionic Acid Substituents

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Summary. The ^{1}H NMR spectra of Biliverdin IXα and Mesobiliverdin XIIIα were studied in order to explain their low resolution in $CDCl_3/CD_3OD$ solvent mixtures. The small T_2 and T_1 values of these ^{1}H NMR spectra are probably due to aggregate formation promoted by the presence of the free propionic acid substituents.

Keywords. Aggregates; Bile pigments; Carboxylic acid association; Micelles.

^{1}H-NMR-Spektren von mit freien Propionsäureresten substituierten Biliverdinen

Zusammenfassung. Es wurden die ^{1}H-NMR-Spektren von Biliverdin IXα und Mesobiliverdin XIIIα untersucht. In $CDCl_3/CD_3OD$-Mischungen erhält man eine schlechte Auflösung. Die niedrigen T_1- und T_2-Werte lassen sich durch eine Aggregatbildung erklären, die durch die freien Propionsäuregruppen verursacht wird.

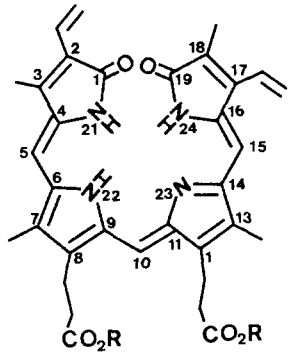
Introduction

As it has been pointed out by a reviewer, the NMR spectra of biliverdins with free propionic acid substituents are "conspicuously unavailable" [1]. To our knowledge, only the ^{1}H NMR spectrum (100 MHz) of biliverdin IXα in *DMSO-d_6* has been reported [1a]. Biliverdins with free propionic acid substituents are slightly soluble in chloroform, but soluble in $CDCl_3/CD_3OD$ or CD_2Cl_2/CD_3OD mixtures at such concentrations that ^{1}H NMR spectra in high field instruments should be easy to obtain. As we show here, the recorded spectra (^{1}H NMR: 200 MHz) in the above mentioned solvent mixtures have a very poor resolution and are not easily interpreted; on the other hand, running the NMR spectra of biliverdins with free carboxylic acid groups in *DMSO-d_6* does not offer any remarkable problem: some broadening is observed due to the well known characteristics of *DMSO* as NMR-solvent.

Results and Discussion

Biliverdin IXα (**1a**) and mesobiliverdin XIIIα (**2a**) in $CDCl_3/CD_3OD$ or CD_2Cl_2/CD_3OD solvent mixtures show ^{1}H NMR spectra with broad signals, some

of them being so broad, that at first sight the obtained spectra could be attributed to very impure samples or to other types of compounds. However, the same samples after solvent evaporation afford good quality spectra in *DMSO-d_6*. Such a low resolution cannot be attributed to the presence of insoluble particles because the sample solutions were filtered through a Millipore (FA) 1 μm pore size filter.



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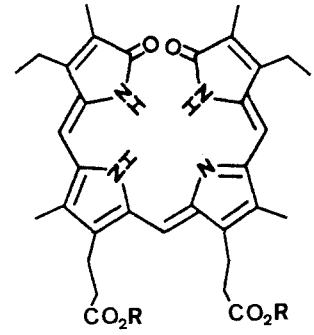

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	R
a	H
b	CH_3

Although dilution improves resolution, it does not improve it to normal quality standards. However, good resolution spectra can be obtained on heating: for $8 \cdot 10^{-3}$ mol l^{-1} **1a** and **2a** in $CDCl_3/CD_3OD$ 4:1, a temperature above 50°C is enough to get spectra with normal resolution standards (see Fig. 1).

On the other hand, the chemical shift of some signals is concentration dependent. Fig. 2 shows the signals having a higher variation of chemical shift with concentration, in $CDCl_3/CD_3OD$ 4:1 solutions. The most affected are H–C10 and CH_3–C(18 or 2/18).

Very characteristic in these spectra is the broadness of the H–C10 singlet signal, which is much higher than the signal of H–C5/H–C15 (see Fig. 1). For example, the H–C10 signal of mesobiliverdin XIIIα (**2a**) at very high dilution ($2 \cdot 10^{-3}$ mol l^{-1}) shows a $\nu_{1/2}$ of 5–6 Hz. Nevertheless, this is a small value compared e.g. to the ca. 40 Hz at a concentration of $38 \cdot 10^{-3}$ mol l^{-1}. Not only the T_2 values are concentration dependent: as it is shown in Table 1 for **2a**, T_1 also changes strongly with concentration: the T_1 values at relatively high concentration of **2a** are very different from the corresponding values of its dimethyl ester (**2b**) under the same experimental conditions; but **2b** shows T_1 values very similar to those reported in the literature [2] for other biliverdins lacking the free propionic acid groups (e.g. **1b**).

In the evaluation of the recorded spectra, sample impurities give additional problems: their relaxation is so different from that of the biliverdin free acid that the relative heights of their signals may induce an error.

The presence of a small proportion of the less stable (*Z,Z,E*)-isomer cannot be excluded: in the case of **2a**, which affords a spectrum simpler than that of **1a**, the chemical shift of the small sharp singlet that appears at 6.1 ppm [H–C15, (*Z,Z,E*)-isomer? [3]], and the corresponding splitting observed for some of the remaining signals could agree with this interpretation. In fact, the signals that show such a splitting are those having a significant chemical shift difference between the (*Z,Z,Z*)- and (*Z,Z,E*)-isomers according to the literature. The variable temperature experi-

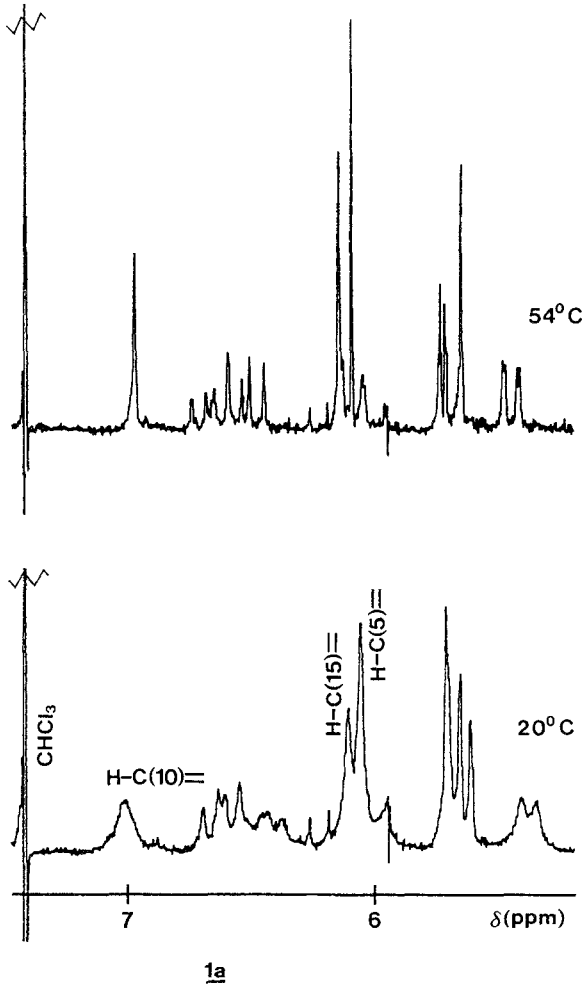

Fig. 1 a

Fig. 1. Partial ^{1}H NMR spectra (200 MHz) of **1 a** and **2 a** in $CDCl_3$: CD_3OD (4 : 1). 8 mmol l^{-1} solutions at 20°C and 54°C

Table 1. T_1 values (inversion-recovery method) in $CDCl_3$: CD_3OD (4 : 1) for mesobiliverdin XIIIα (**2 a**) and its dimethyl ester (**2 b**) at normal work concentrations and for **2 a** at high dilution

	Spin-lattice relaxation times (T_1, s)		
	2 b[a] 50 mmol l^{-1}	**2 a** 59 mmol l^{-1}	**2 a** 2 mmol l^{-1}
H–C(10)=	0.50 ± 0.01	≅ 0.13	0.41 ± 0.03
H–C(5,15)=	0.72 ± 0.02	0.13 + 0.01	0.68 ± 0.03
CH_3OOC—	1.21 ± 0.05		
—OOC—CH_2—$C\bar{H}_2$—C(8,12)	0.28 ± 0.05	≅ 0.10	0.21 ± 0.01
—OOC—$C\bar{H}_2$—CH_2—C(8,12)	0.42 ± 0.02	≅ 0.11	0.27 ± 0.01
CH_3—$C\bar{H}_2$—C(3,17)	0.29 ± 0.03		0.25 ± 0.01
CH_3—C(7,13)	0.48 ± 0.03	0.13 ± 0.02	0.44 ± 0.03
CH_3—C(2,18)	0.53 ± 0.04	0.18 ± 0.01	0.49 ± 0.04
$C\bar{H}_3$—CH_2—C(3,17)	0.72 ± 0.02	0.19 ± 0.01	0.62 ± 0.04

[a] Similar values are described in the literature for **1 b** in $CDCl_3$

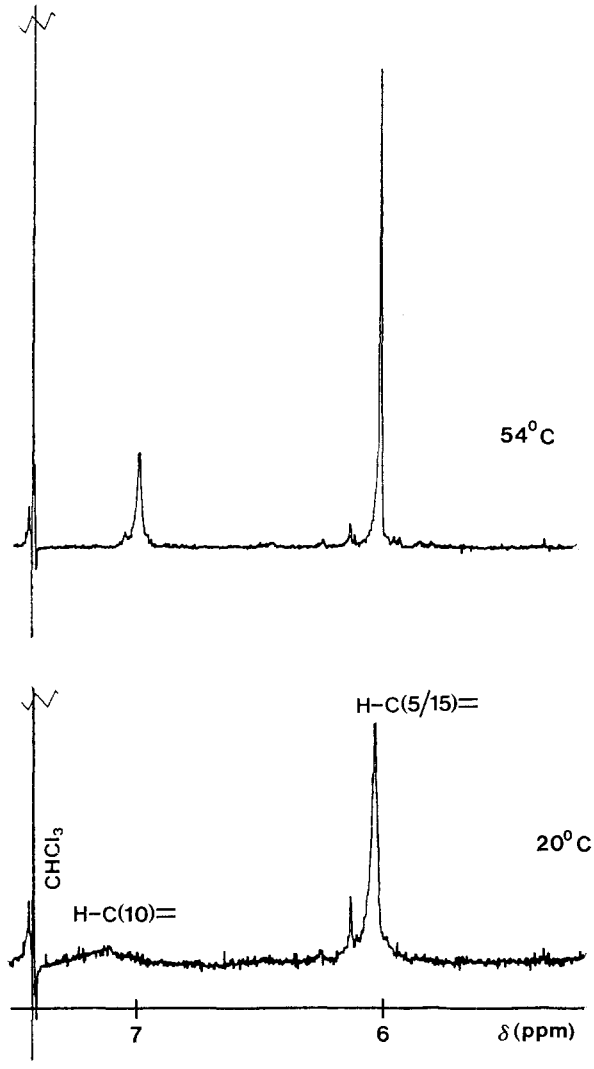

Fig. 1 b

2a

ments and the changes observed in the spectra with dilution show a very different behaviour for the signals of the (*Z*,*Z*,*Z*)-isomer with a strong change of their relative heights with respect to those of the hypothetical (*Z*,*Z*,*E*)-isomer (which does not show significative changes on T_2).

All these results point to some type of aggregation: In fact, N*O*ESY pulse sequences applied on **2 a** ($60 \cdot 10^{-3}$ mol l^{-1} in $CDCl_3/CD_3OD$ 4 : 1) do not give any information about the well known N*O*Es in biliverdins [3]. In contrast, a R*O*ESY (Rotating Frame Overhauser Effect Spectroscopy) pulse sequence, which is applied to study N*O*E and exchange processes on molecules in which the extreme narrowing condition does no hold (i.e. relatively large molecules, which correlation time τ_c is such that $1/\tau_c \cong \omega_0$), allows to see the cross peaks expected for the helical form of (*Z*,*Z*,*Z*)-biliverdins [3], except for those corresponding to the H–C10 signal, which owing to its broadness does not afford reliable cross peaks. Due to the molecular weight, the fact of not fitting the extreme narrowing condition in a 200 MHz spectrometer suggests the presence of at least a dimeric structure: e.g. a bilatriene

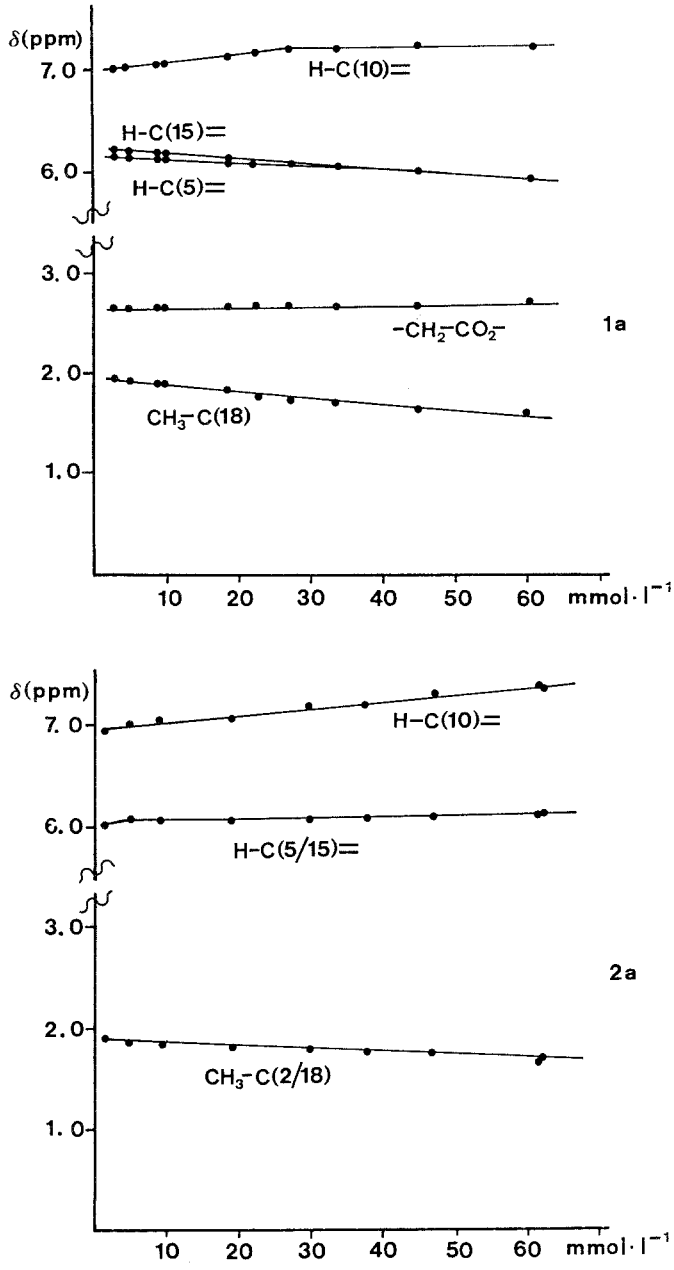

Fig. 2. Chemical shift dependence on concentration of some of the signals of the ^{1}H NMR spectra of **1a** and **2a** in $CDCl_3$: CD_3OD (4 : 1) at room temperature; the signals not shown in the figure have a smaller concentration dependence

with similar molecular weight has been studied both by N*O*ESY and R*O*ESY with a 360 MHz instrument [4].

With such aggregates being specific for the free acids, the carboxylic acid group must play an important role in their formation. It has already been demonstrated by vapour pressure osmometry that in $CHCl_3$ biliverdins with a free propionic acid substituent at C8 tend to be dimeric [5]. A second, much weaker interaction that could play an additional role in the formation of any type of aggregate is

a donor-acceptor intermolecular interaction between the π-systems of the bilatrienes. In this context it must be mentioned that in biliverdins, HOMO and LUMO are located in different halves of the molecule, their concrete location being determined by the N22–N23 tautomerism [6]. Although such types of interactions should be observed in the electronic spectra, at the concentrations used it is not easy to obtain reliable UV/Vis spectra of biliverdins. However, as it is well known by the experimental chemist but scarcely reported in the literature (see [1] as an exception), small solvatochromic and thermochromic effects are often visually observed in biliverdins.

When running the ^{13}C NMR spectra of **1 a** in these solvent mixtures, some difficulties were encountered due to its low solubility. The signals obtained for **1 a** and **2 a** appear at chemical shifts very close to those described for the dimethyl esters of some biliverdins [7, 8]. As in the case of the ^1H NMR spectra, the signals are broad, especially some of the signals of the quaternary sp^2 carbon atoms of the bilatriene π system. Except from the lower solubility in CH_3OH, there is no difference between the spectra of **1 a** in $CDCl_3/CD_3OD$ or in $CDCl_3/CH_3OH$: excluding any interpretation based on an isotope effect upon the tautomer exchange. Also—as in the case of ^1H NMR—we have obtained a "normal" ^{13}C NMR spectrum of **2 a** by running the same sample in *DMSO-d_6*.

It is remarkable that with H–C10 being the most prominent signal (T_2) in the ^1H NMR spectra, the corresponding C10 signal in the ^{13}C NMR spectra was not the most affected. This characteristic of the NMR spectra may also be explained by the presence of aggregates. The propionic acid chains are "locked" because of the formation of the aggregate and must have a low mobility. A long τ_c and the dipole-dipole relaxation between H–C10 and the "fixed" methylene group at C8 and C12 would explain the small T_2 values of these signals. In this sense, it must be pointed out that for both **1 a** and **2 a** the signals corresponding to this methylene group are broader than those of the other methylenes in the molecule. Despite of the N22–N23 tautomerization process and the "quasi-planar" interconversion of the helical enantiomeric conformers, if the torsion around the C9–C10 single bond (the second favoured path in the interconversion between helices [9]) is locked by the "fixed" propionic acid chains, the H–C10 motion will be very small.

Acknowledgement

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Experimental

Biliverdin IXα (**1 a**), mesobiliverdin XIIIα (**2 a**) and mesobiliverdin XIIIα dimethyl ester (**2 b**) are described in the literature [10–12].

The NMR spectra were recorded in a Varian XL-200 (Advance 6800 Y based data system) instrument (200 MHz, ^1H NMR and 50.3 MHz, ^{13}C NMR) using *TMS* as internal reference. For N*O*ESY and R*O*ESY experiments the samples were degassed using an argon stream.

^1H NMR spin-lattice relaxation times (T_1: see Table 1) were measured by the $(180°\text{-}\tau\text{-}90°\text{-}t)x$ inversion-recovery sequence with an average of 64 free induction decays: delay between sequences was 12 s; values of τ between 0.023 and 12 s were "arrayed" using the XL-200 T_1 program.

^1H NMR N*O*ESY experiments [13, 14] on **2 a** were performed at the following conditions: the mixing times were 90 and 150 ms, 64 transients were accumulated for 256 values of evolution period; a spectral width of 2936 Hz in both dimensions, and a delay of 1.2 s was employed. A 2K × 1K data matrix was used.

^{1}H NMR ROESY experiments [15] on **2a** were performed at the following conditions: 2D spectral width was 2 936 Hz; mixing times of 90 and 150 ms were used; the 90° pulse was 7.6 μs, the spin-locking pulse was 2.5 μs; 2 × 256 increments of evolution time, and a 2K × 1K data matrix was used. The interactions found were analogous to those described in the literature [3] for non free acid biliverdins (i.e. their methyl esters). No reliable cross peaks were obtained on H–C10, probably due to the flatness of this signal.

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