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The Structure of "Photobilirubin"

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Anaerobic irradiation of bilibrubin in the region of the main absorption band produces the diastereomers of (*Z,E*)- and (*E,Z*)-configurations with high quantum yield. The structure of these "photobilirubins" is proven by a chemical correlation with the corresponding diastereomers of biliverdindimethylester characterised before. Their ^{1}H-NMR spectroscopic features have been recorded and are discussed.

[Keywords: (Z,Z)-, (Z,E)- and (E,Z)-Bilirubin; Photobilirubin; Neonatal jaundice; Phototherapy]

Die Struktur der „Photobilirubine"

Anaerobe Bestrahlung von Bilirubin im Gebiet der Hauptabsorptionsbande ergibt in hoher Quantenausbeute die Diastereomeren der Konfigurationen (*Z,E*) und (*E,Z*). Die Struktur dieser „Photobilirubine" wird durch eine chemische Korrelation mit den entsprechenden bekannten Diastereomeren des Biliverdindimethylesters bewiesen. Die ^{1}H-NMR-Daten der Diastereomeren werden diskutiert.

Introduction

By accident it has been found that neonatal jaundice may be alleviated or even cured by phototherapy[1]. This fact prompted a vivid investigation of bilirubin chemistry and photochemistry. The possible routes of photooxidative bilirubin degradation and the photoaddition of suitable substrates to vinylic side chains or even the formation of positional isomers are well documented[2–4]. Later it became clear that these reactions proceed with rather low quantum yields or possibly at wavelengths outside the main absorption region of bilirubin[5].

There is another facet of bilirubin photochemistry which became obvious from investigations of bilirubin partial structure models like

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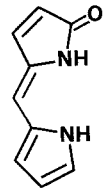
arylmethylidenepyrrolinones[6] and 5-(1*H*)pyrromethenones[7]: Unlike the pyrromethenes (I) which, by very rapid tautomeric processes behave as if they were of C_{2v} symmetry[8], 5-(1*H*)pyrromethenones (II) have an exocyclic double bond at the pyrrolinone ring which is not

I



II

shifted by tautomeric processes. As a consequence stable diastereomers of the two possible configurations (*Z*) and (*E*) could be prepared (based on thermodynamics or photochemically), isolated and characterised[9]. The quantum yield of the corresponding photochemical isomerisation is very high (~ 0.3)[10] and the thermal barrier is sufficient to allow for handling and physical measurements[7]; the barrier is strongly decreased by protons[7].

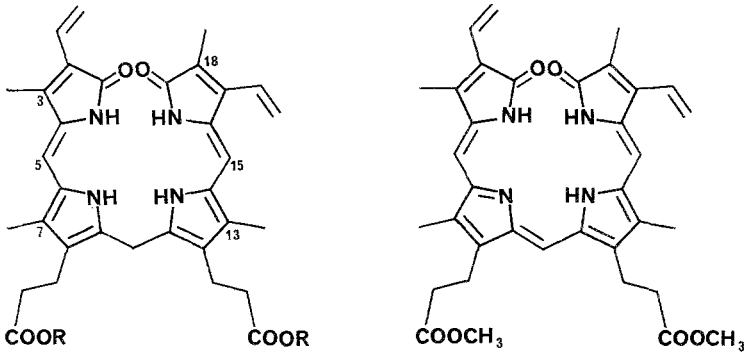
This idea of photoisomerisation at the exocyclic double bond has been thoroughly followed up by several investigators and very interesting aspects of bilirubin photochemistry could be revealed[11–16]. Nevertheless, the proof of structure for the photoisomers of bilirubin which were named "photobilirubin" is lacking up to now.

The motivation and purpose of this paper is to ascertain the structure of the products stemming from anaerobic irradiation of bilirubin using light of wavelengths within the bilirubin absorption band.

Materials and Methods

Commercial bilirubin [(*Z*,*Z*)-**1**; bilirubin-IX-α; Fluka, p.a.] was esterified[17] to yield bilirubindimethylester (*Z*,*Z*)-**2**. Oxidation of **2** afforded biliverdin-dimethylester (*Z*,*Z*,*Z*)-**3** from which the photoisomers (*E*,*Z*,*Z*)-**3** and (*Z*,*Z*,*E*)-**3** were prepared and isolated[18].

Chloroform and methanol (p. a., Merck) were dried over molecular sieves (4 Å), distilled under Ar and degassed by three freeze pump thaw cycles. Immediately before using $CHCl_3$ it was percolated under argon through basic



Al_2O_3, activity I (Merck). The operations were generally performed using a Kodak sodium safety lamp No. 2 and "inactinic glass" vessels (Sovirel).

As light sources a xenon high pressure arc (1 kW) coupled to a high energy monochromator GM 252 (Kratos) using band widths from 5–20 nm (1), a HPL-N-700W-7C Philips lamp (2) and Philips-TL-20W light rods (3) which have an intense line around 440 nm and almost no lines below 400 nm were used.

Irradiations were performed on stirred solutions of **1** and **2** in $CHCl_3$ (concentrations about 10^{-4} mol l^{-1}; 0,5–250 ml, degassed: three freeze-pump-thaw-cycles) contained in water cooled (15 °C) Pyrex vessels. Photoequilibrium as judged by absorption difference spectroscopy and ^{1}H-NMR was reached within 1 h (1) and 15 min (2), (3) resp. The resulting solutions were cooled to −80 °C and the solvent evaporated at 10^{-3} mbar using a trap cooled with liqu. N_2. The remaining solid was then dissolved in NMR- or HPLC-solvents or extracted with CH_3OH [2 min sonication, inert filtration from (*Z,Z*)-**1**] to yield enriched photobilirubin after evaporation at −100 °C.

^{1}H-NMR spectrometric measurements were performed on a Bruker WM-360 instrument at 300 K. $CDCl_3$ (99.96%) degassed and percolated through basic Al_2O_3 immediately before use, CD_3OD (99.96%) and *DMSO-d*$_6$ (99.8%) degassed and dried over molecular sieves (3 Å) were used as solvents (*TMS* served as internal reference). Solutions (10^{-3} — 10^{-4} mol l^{-1}) were degassed by two freeze-pump-thaw-cycles. *NOE*-difference-spectra were recorded using the appropriate microprogram of the Aspect 2000 data system. An observation pulse angle of 25° (2 μs) was used throughout. Approximately 1000 transients were accumulated typically using 32 K or 64 K datapoints with quadrature detection at a spectral width of 4 000 Hz or 5 500 Hz resp. Amber NMR-tubes (Wilmad, 5 mm) were used for the spectra of the photosensitive samples.

HPLC was performed on the Varian Model 5000 and Perkin Elmer series 2 liquid chromatographs using Perkin Elmer LC-75 and Varian LC 50 detectors.

As stationary phases Lichrosorb SI 100 (7 μ, Merck) and RP-8 (Merck) were used in columns 250 mm long and having an internal diameter of 4.6 mm. The solvents (toluene, dimethylformamide, and methanol) were degassed by N_2-bubbling. Solutions were diluted at least 1:10 with the mobile phase where the solvent was not identical with the mobile phase to avoid "ghost" peaks. TLC was performed as given in Ref.[11,15].

Transformation procedure for **1** over **2** to **3**: Photobilirubin or methanolic photobilirubin extract evaporated to dryness was dissolved in $CHCl_3$. According to its content of rubinoid pigment (estimated from spectrophotometric measurement) a 50% excess of etheric, freshly distilled CH_2N_2 solution was added at once and after 10 min stirring at 20 °C, CH_2N_2 was removed by bubbling with argon. The resulting solution was either used for chromatographic determinations, after evaporation (— 80 °C) for ^{1}H-NMR experiments, or immediately for the next step: A 10% excess of chloranil dissolved in benzene or toluene was added and the resulting bluegreen solution evaporated for analytical purposes or used immediatly for chromatographic separations.

Quantum yields were estimated using the *Reinecke* salt actinometer[19].

Results and Discussion

*1. The Photoreaction of (Z,Z)-***1** *— ^{1}H-NMR Features*

On irradiation of (*Z*,*Z*)-**1** in carefully degassed chloroform solutions at wavelengths from 390-470 nm a photostationary state is reached. The composition of this state varies quantitatively within about 10-20% but qualitatively the changes observed by differential absorption spectroscopy, HPLC on reversed phase and ^{1}H-NMR are independent of irradiation wavelength within the absorption band. The quantum yield of this reaction is found to be 0.17 ± 0.02 which is quite the same order which has been found for **1** bound to albumin[16]. As the data on differential absorption spectroscopy[13], TLC[13] and HPLC[20] are in agreement with results published already and reproduced under the conditions given there, we will concentrate on the ^{1}H-NMR results which became accessible only by the enhanced sensitivity and resolution of high field instruments: Fig. 1 gives an example of what is happening in the region of the methyl group signals on irradiation of (*Z*,*Z*)-**1**. Several new signals evolve until the photostationary state is reached. Such mixture spectra remain unchanged up to two days at room temperature but revert to the initial (*Z*,*Z*)-**1** spectra on prolonged standing or heating. The same could be observed by TLC, HPLC and differential absorption spectroscopy, indicating clean thermal reversion under carefully controlled environments.

Chromatographic isolation of the pure "photobilirubin" fraction failed due to partial reversion to the (*Z*,*Z*)-diastereomer in the course of the separation procedure. Therefore we resorted to a much simpler extraction step: (*Z*,*Z*)-**1** is practically insoluble in methanol[21] or

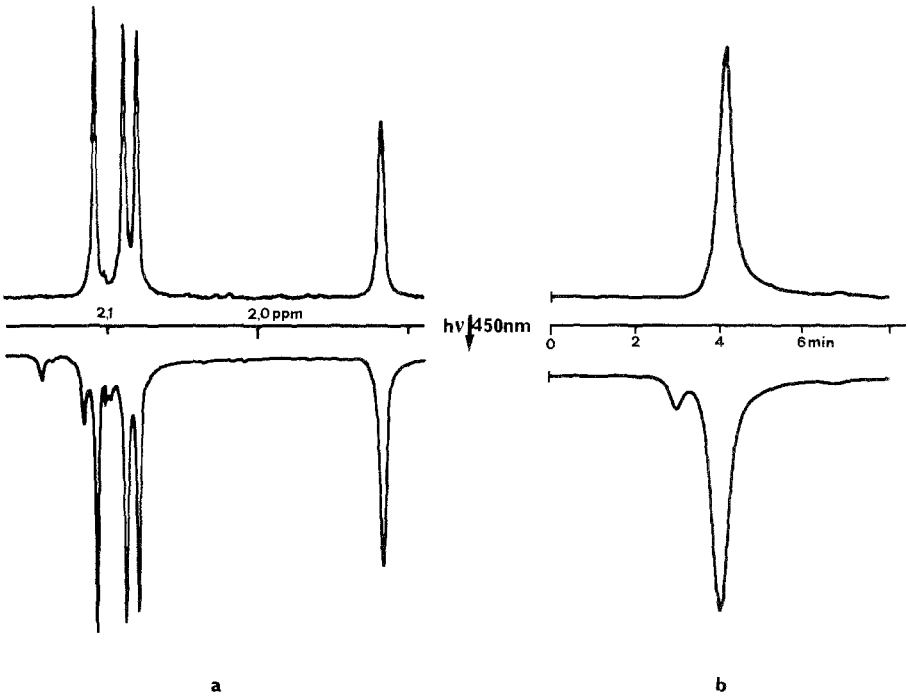


Fig. 1. ^{1}H-NMR spectra of (*Z*,*Z*)-**1** in $CDCl_3$ (360 MHz, methyl group region) before and after irradiation (450 nm) until a photostationary state is reached (*a*) and HPLC traces [RP-8, CH_3OH/25% aqu. NH_3/H_2O = 72.3/0.7/27 (*v*,*v*,*v*), flow 2 ml min^{-1}, detection at 450 nm] (*b*)

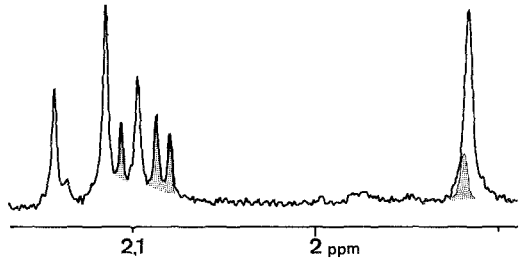


Fig. 2. ^{1}H-NMR spectrum of a methanolic "photobilirubin" extract in $CDCl_3$ (360 MHz, methyl group region); signals of accompanying (*Z*,*Z*)-**1** are shaded

acetone whereas the photobilirubin fraction is easily soluble in these solvents. Fig. 2 presents the ^{1}H-NMR data of such a methanol extract. From comparison with spectra of the type of Fig. **1**, as well as from chromatographic behaviour and reversion characteristics the photobilirubin remains unchanged by the extraction procedure.

Moreover, under a variety of irradiation conditions (e.g. adsorbed to albumin in buffer solutions, using *DMSO* as solvent) the same feature of ^{1}H-NMR signals were obtained after comparable extraction steps.

2. Structural Analysis by Chemical Correlation

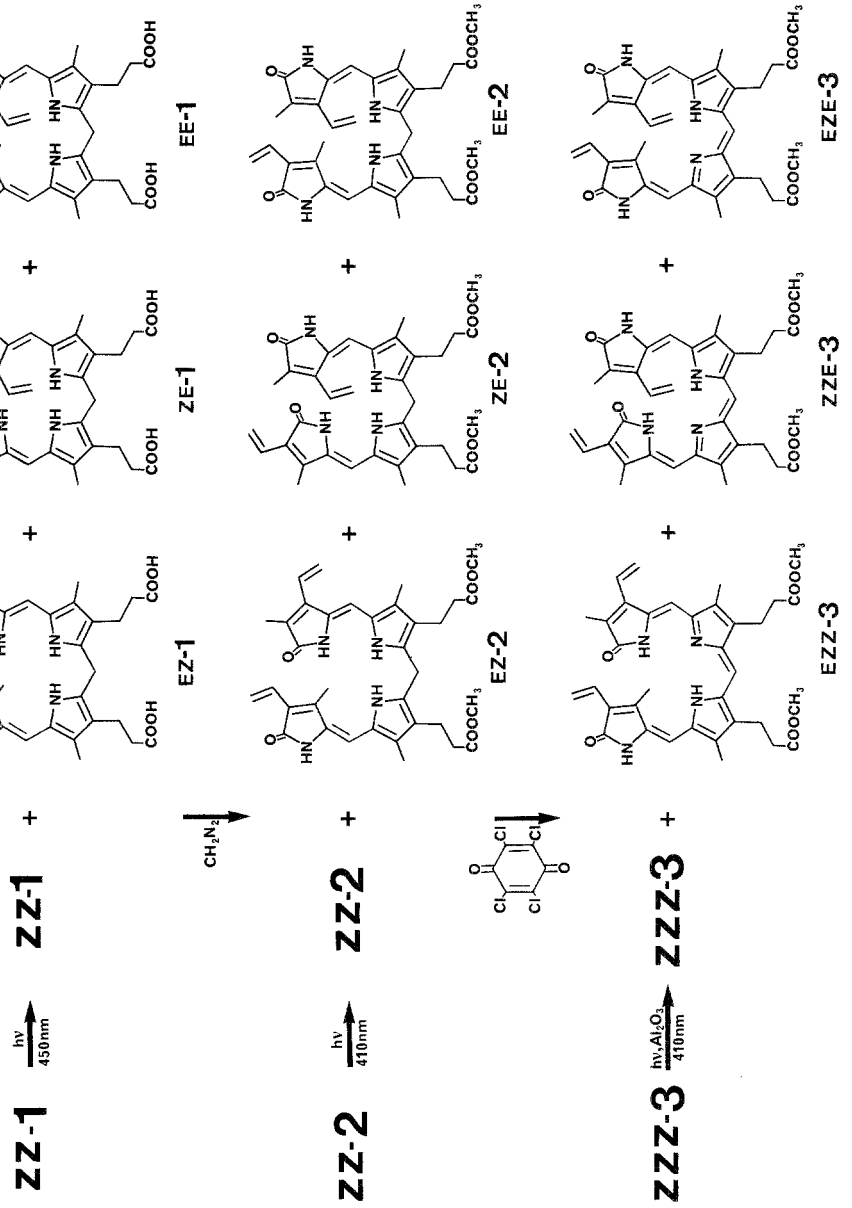
Having in mind the hypothesis that "photobilirubin" is the product of anaerobic photoisomerisation at the exocyclic double bonds of the lactam rings [which yield the corresponding (*Z*,*E*)-, (*E*,*Z*)- and (*E*,*E*) diastereomers] a chemical correlation to defined derivatives would be a means of establishing their structures and configurations.

The biliverdindimethylester diastereomers of (*Z*,*Z*,*E*)- and (*E*,*Z*,*Z*)-configuration were isolated, characterised and their configurations assigned recently[18]. For a chemical correlation of "photobilirubin" with these diastereomers an sterification—oxidation procedure is necessary therefore. By esterifying first, there is also the possibility to structurally correlate even the photoisomers of bilirubindimethylester characterised chromatographically before[11]. Scheme **1** presents this transformations. In the first step the photoisomerisation products of (*Z*,*Z*,*Z*)-**3**, namely (*Z*,*Z*,*E*)-**3** and (*E*,*Z*,*Z*)-**3** could be isolated and characterised[18]. Although there are hints that some (*E*,*Z*,*E*)-**3** is produced as well, we were not able to purify it for a structural characterisation, but the formation and structure of this product is corroborated further by comparison with the (*E*,*Z*,*E*)-aetiobiliverdin-IV-γ which could be isolated and characterised[22].

In the second step (*Z*,*Z*)-**1** was irradiated and the methanol extract prepared as given above. Esterification with diazomethane as the third step afforded a sample which showed the same components produced by irradiation of (*Z*,*Z*)-**2** according to Ref.[11] by HPLC. Step four: oxidation of this mixture with chloranil produced the corresponding HPLC pattern which is also obtained from (*Z*,*Z*,*Z*)-**3** photoisomerisation. The photoisomers from the oxidation procedure could be isolated as well by TLC and their identity proven by comparison of ^{1}H-NMR and UV-VIS spectral data of products and authentical samples[18]. Thereby the structure of "photobilirubin" being a mixture of (*Z*,*E*)- and (*E*,*Z*)-**1** as well as the one for "photobilirubindimethylester" representing (*Z*,*E*)- and (*E*,*Z*)-**2** is established unequivocally.

A set of typical HPLC traces is shown in Fig. 3 for the transformation steps. Note the peaks of apolar material at the esterification procedure stemming from the known lactim ether formation[23]. They are destroyed in the oxidation step. It should also be noted that the HPLC assignments were achieved from a variety of experiments by

Schema 1


ZZ-1
hν 450nm
ZZ-1 + EE-1 + ZE-1 + EZ-1
CH2N2
ZZ-2 + EE-2 + ZE-2 + EZ-2
hν 410nm
ZZ-2
Cl Cl O O Cl Cl
ZZ-3 + EZZ-3 + ZZE-3 + EZE-3
hν,Al2O3 410nm
ZZZ-3

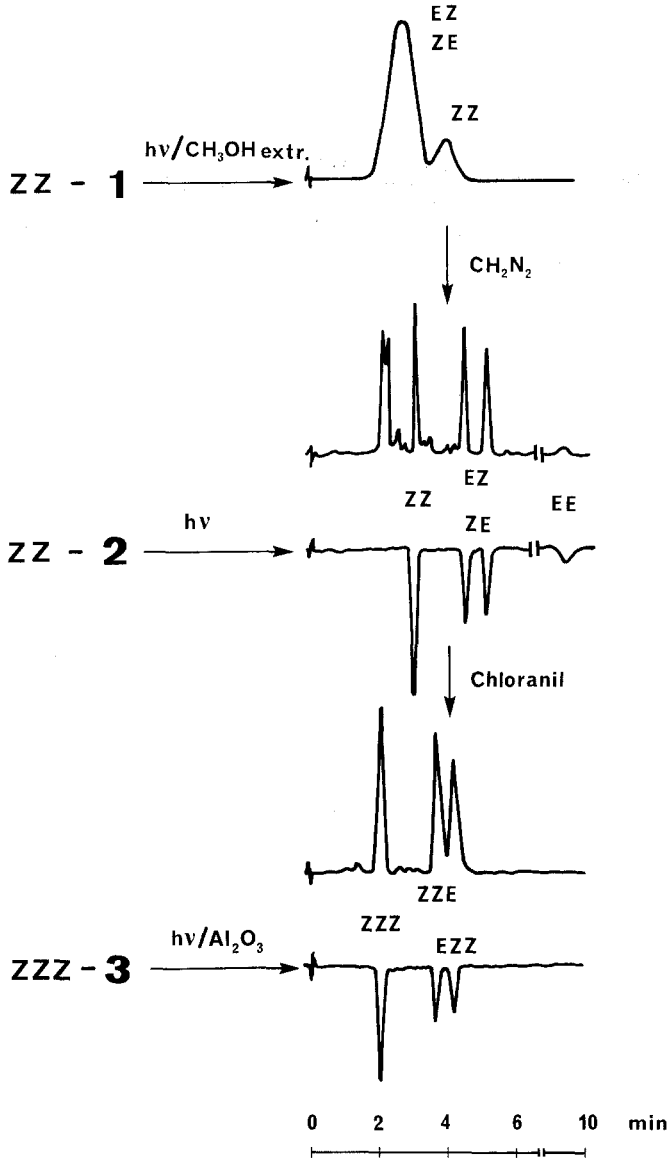


Fig. 3. HPLC traces for the chemical correlation steps from "photobilirubin" methanol extract to the biliverdindimethylester diastereomers via the corresponding bilirubindimethylester isomers [**1**: RP-8, $CH_3OH/25\%$ aqu. $NH_3/H_2O = 27.3/0.7/27$ (*v,v,v*); **2**: SI-100, toluene/dimethylformamide = 93/7 (*v,v*); **3**: Si-100, toluene/dimethylformamide = 96/4 (*v,v*); flow 2 ml min^{-1} throughout, detection wavelength at the absorption maxima of the corresponding stable diastereomer]

isolating and reinjecting single peaks, comparisons to mixed injections as well as to authentic samples and carrying (*Z*,*Z*)-**1** and (*Z*,*Z*)-**2** without irradiation through the correlation steps.

3. ^{1}H-NMR-spectroscopic Assignments for "Photobilirubin"

As there is obviously strong overlapping of ^{1}H-NMR peaks of the two photoisomers as judged from an inspection of Fig. 2, admixture of *DMSO-d_6* to the $CDCl_3$ solution led to shifts of the signals. Although all eight methyl resonances of the photoisomers could be resolved thereby assignments were complicated by disturbing solvent effects on the *NOE* measurements. Therefore CD_3OD was used as solvent after a double methanol extraction step which removed all (*Z*,*Z*)-**1**. The reversion of the photoisomers in methanol although faster as in chloroform is still sufficiently slow enough to allow the measurements, moreover, one of the photoisomers reverts back a little faster than the other one providing a simple means of discriminating between the two sets of signals. Fig. 4 presents the ^{1}H-NMR spectrum of the two photoisomers in CD_3OD together with the assignments from decoupling and *NOE* difference spectra: In case of (5*Z*,15*E*)-**1** a coupling between CH_3-18 and CH-15 is observed (compare[18]), *NOE*s are found from CH_3-3 as well as CH_3-7 on CH-5 yielding the stereochemical as well as the signal assignment. Due to only a slight difference between the two *NOE*s the CH_3-3 and -7 assignments may be interchanged. For (5*E*,15*Z*)-**1** there is a small coupling between CH_3-18 and CH-15 as well as a small *NOE* between CH_3-7 and CH-5 and a negative *NOE* between CH_3-13 and CH-15. The latter has also been observed in other "linear" spin systems[24].

It should be noted that using this approach methyl and methine signals of (*Z*,*Z*)-**1** could be assigned as well which could not be achieved before[25]: CH_3-18 at 1.947 ppm ($CDCl_3$, 360 MHz) is coupled to CH-15 at 6.139 ppm (*J* appr. 0.4 Hz). Irradiation of CH_3-13 at 2.086 ppm produces a *NOE* at this latter methin signal. *NOE*s are observed as well from CH_3-3 (2.076 ppm) and CH_3-7 (2.104 ppm) on CH-5 at 6.06 ppm, but a discrimination between the two signals is based only on the somewhat stronger *NOE* for CH_3-3. In addition these *NOE* data indicate a *syn*-(*Z*)-*syn*-(*Z*) conformation.

This *NOE* behaviour of the "left" side of (*Z*,*Z*)-**1** is nicely found for the corresponding fragment of (*Z*,*E*)-**1**. These measurements discussed above therefore not only provide the assignments for methyl and methine signals of the two photodiastereomers but as well present additional evidence for the structure and stereochemistry of these derivatives.

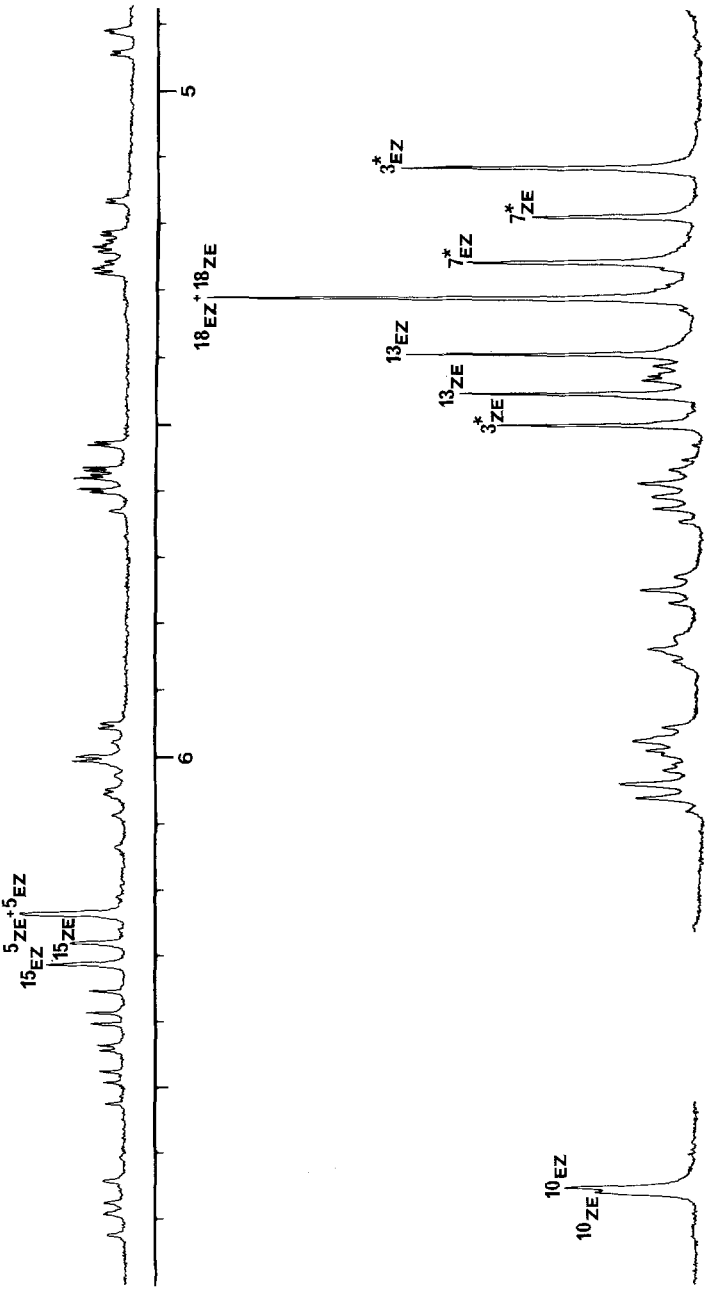


Fig. 4. ^{1}H-NMR spectrum of (*Z*,*E*)-**1** + (*E*,*Z*)-**1** in CD_3OD (360 MHz). Assignments marked with an asterisk are incertain and may be interchanged within the corresponding diastereomer. The signals of the four propionic acid side chains (2.0-2.75 ppm) and the four vinyl groups (4.9-6.8 ppm) could not be assigned with confidence due to overlap

Conclusions

From the results given above it can be concluded that irradiation of bilirubin [(*Z*,*Z*)-**1**] under anaerobic conditions in the region of the main absorption band yields the photodiasteromers of configurations (*Z*,*E*) and (*E*,*Z*) and presumably a minor amount of the (*E*,*E*) isomer with high quantum efficiency.

On the correlation of this conclusion with results of published photochemical results one might argue as follows: The products obtained are identical to the "photobilirubin" obtained by *Lightner* and *McDonagh*[13], *Onishi*[20] and possibly the photobilirubins IA and IB (or at least one of them) of *Stoll*[15] as judged form the repetition of their experiments under comparable conditions. Although we were not able to separate the isomers IA and IB [and to get them free of (*Z*,*Z*)-**1**] of *Stoll*[15] the corresponding fraction which has been prepared according to their procedure prooved to be identical with ours*. Therefore the main photoproducts of *Lightner*[13] as well as of *Lamola*[16] and *Onishi*[20] which were analyzed by HPLC and ^{1}H-NMR under the conditions given above seem to be identical with the primary "labile" product of *Stoll*[15].

Provided the irradiation is executed under anarerobic conditions and at appropriate wave lengths within the absorption band using only doses sufficient to reach photoequilibrium for the highly efficient photoisomerisation the main product will be a mixture of (*Z*,*E*)- and (*E*,*Z*)-**1** and presumably a minor quantity of (*E*,*E*)-**1** regardless of the environmental conditions (solvent, adsorption, etc.).

By the same arguments the photobilirubin-dimethylesters are shown to be diastereomers as well and the configurations (*Z*,*E*) and (*E*,*Z*) are assigned to them as it has been done tentatively before[11].

Acknowledgements

The skilled technical assistance of Ing. *S. Wansch* is gratefully acknowledged.

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