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THE DIAZO REACTION OF BILIRUBIN: STRUCTURE OF THE YELLOW PRODUCTS

(STUDIES ON PLANT BILE PIGMENTS—14[1])

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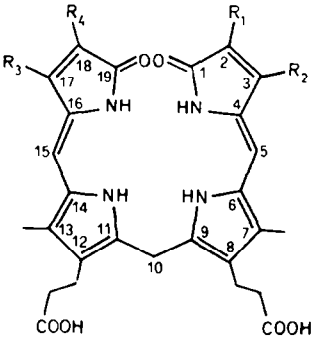
Abstract —The reaction of bilirubins with aromatic diazonium salts in alcoholic solvents leads to an equimolar mixture of two types of products. One is the well-known 9-azopyrromethenone. The other is a yellow product ($\lambda_{max} \approx 420$ nm) identified as 9-alkoxymethylpyrromethenone, the alkoxy-substituent being derived from the solvent. Thus, reaction of the symmetrically substituted bilirubins IIIα (**1c**) and XIIIα (**1b**) in methanol with diazotized sulfanilic acid yields one mole of the azopigments (**4b** and **4a**), respectively, and one mole of the corresponding 9-methoxymethylpyrromethenones (**2b** and **2a**). Bilirubin IXα (**1a**) consequently yields a mixture of all four products. The two resulting 9-methoxymethylpyrromethenones were separated by chromatography and identified as **2a** and **2b**. They can react further with the diazonium salts to give the corresponding 9-azopyrromethenones, but the reaction is much slower than that of bilirubin, which explains the observed product distribution. These results are discussed in relation to earlier work.

ALMOST a hundred years ago Ehrlich[2] discovered the formation of a violet colour when a solution of bilirubin was treated with diazotized sulfanilic acid. Fischer and Haberland[3] demonstrated that bilirubin was cleaved around the central methylene bridge by the aromatic diazonium salts thus forming a pair of isomeric azodipyrroles. It was soon recognized that this reaction was of practical use for the qualitative and quantitative determination of bilirubin in body fluids[4] and today it is used widely for this purpose. It is also used as a research tool for the structural investigation of naturally occurring bilirubins and their conjugates.[5]

We recently used the diazo reaction as a novel degradation technique for biliproteins.[6] In a two-step reaction the verdin type chromophore of denatured phycocyanin was reduced with $NaBH_4$ to the corresponding rubin and the "phycorubin" formed was subsequently treated with aromatic diazonium salt. The amount of azopigment found, however, never exceeded 50% of the theoretically expected value, if a complete conversion of the "phycorubin" to azopigments was assumed. Instead a second product with $\lambda_{max} = 400$ nm was observed.[6] It was shown that this was not due to an unusual reactivity of the phycorubin chromophore since bilirubin IXα gave similar results. To establish the nature of the yellow products, we have now studied the reaction under different conditions.

Spectroscopic studies. When bilirubin IXα (**1a**, concentration range 14–23 μM) in methanol or ethanol containing a trace of dimethylsulfoxide was treated with diazotized sulfanilic acid (aqueous solution in 0.1 N HCl), the colour changed from yellow to orange-red. The bilirubin band at 451 nm (MeOH) or 446 nm (EtOH) disappeared, and new bands arose instead at 518, 415 and 330 nm (MeOH), or 524, 420 and 330 nm (EtOH). The 330 nm band was only resolved in the presence of a *small* excess of diazo reagent, due to the interfering absorption of the latter at 268 and 297 nm.[7] Isobestic points were observed at 487 and 381 nm (MeOH) or 486 and 398 nm (EtOH).

Identical absorption maxima were eventually obtained, irrespective of a 1.2-, 4- or 6-fold molar excess of diazonium salt over bilirubin being used. The reaction was accelerated, however, by an increasing excess of reagent. With only slight excess of diazonium reagent (1.2 molar ratio) it took approximately 30 min to reach the point where no further significant spectral changes could be observed, whereas this part of the reaction was already complete after mixing and recording the spectrum with a 4- or 6-fold excess of diazonium reagent. These rapid spectral changes were followed by a much slower phase, during which the



	R_1	R_2	R_3	R_4
1a:	CH_3	C_2H_3	CH_3	C_2H_3
1b:	CH_3	C_2H_3	C_2H_3	CH_3
1c:	C_2H_3	CH_3	CH_3	C_2H_3

band around 420 nm decreased by 10–15% over a period of 30 min, with a concomitant increase of the band around 520 nm.

For preparative work, the solvent system was changed to methanol : chloroform (95 : 5) to enhance the solubility of bilirubin. The diazo reagent was neutralized immediately before addition, to exclude acid-catalyzed side reactions, e.g. hydrolysis or esterification. Only a 1.5 molar excess of the reagent was used because the azopyrromethenones formed from bilirubin are unstable in the presence of a larger excess of diazo reagent.[8] The spectral changes during a preparative scale reaction of bilirubin IXα (**1a**) (43 μM) reagent was not neutralized prior to its use. They probably contain the methyl esters of **2a, b**.) The R_f of **1a** was 0.84 in this solvent system. Bilirubin IIIα (**1c**, $R_f = 0.91$) gave only the more mobile component ($R_f = 0.28$) while bilirubin XIIIα (**1b**, $R_f = 0.79$) gave only the less mobile one ($R_f = 0.24$). The spectroscopic yield of the yellow pigments from **1a** was 93%. Since the two yellow pigments from **1a** were difficult to separate by preparative thin layer chromatography, the pure pigments were prepared individually from bilirubin IIIα and bilirubin XIIIα, the latter compound being available in modest amounts by the recent elegant procedure of Monti and Manitto.[10]

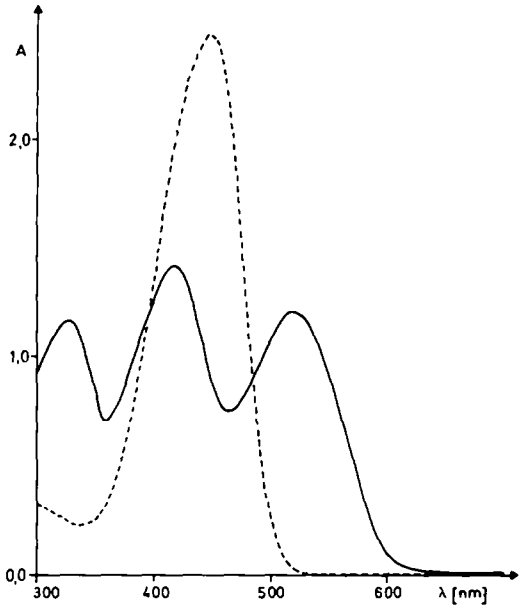


Fig. 1. Diazo-reaction of bilirubin IXα (**1a**). ----: **1a** (43 μM) in methanol: chloroform = 95:5. ——: Reaction mixture after addition of diazotized sulfanilic acid (62 μM) and 15 min reaction at ambient temperature. The diazonium reagent was neutralized with 0.1 M NaOH prior to addition. The bands at 520 and 328 nm are due to the 9-azopyrromethenones (**4a, b**), the one at 416 nm to the 9-methoxymethylpyrromethenones (**2a, b**).

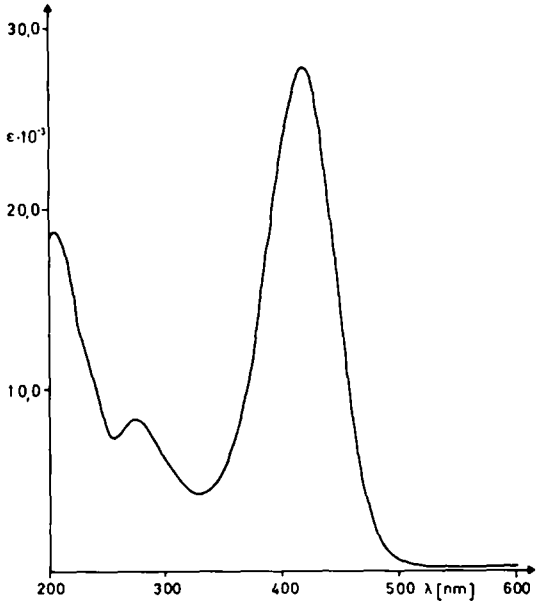
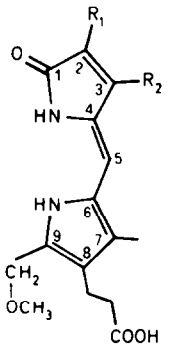


Fig. 2. Electronic absorption spectrum (methanol) of a mixture of the isomeric 9-methoxymethylpyrromethenones, **2a** and **2b**.

are shown in Fig. 1. The product mixture had absorption maxima at 520, 416 and 328 nm. Bilirubins XIIIα (**1b**) (λ_{max} = 445 nm) and IIIα (**1c**) (λ_{max} = 452 nm) reacted similarly, absorption maxima appearing at 516, 416, 325 nm, and at 526, 417, 330 nm, respectively.

Isolation and structures of the reaction products. The mixture obtained from bilirubin IXα and diazotized sulfanilic acid was partitioned between chloroform and water. The aqueous phase contained a violet pigment (λ_{max} = 515, 325, 278 nm). These absorption maxima are typical[9] of the well-known 9-azopyrromethenones, which are rendered water soluble in this case by the sulfonic acid substituent, as in structures (**4a, b**). The yellow chloroform phase (λ_{max} = 418 nm) gave two yellow spots (R_f = 0.24 and 0.28) on analytical thin layer chromatography (silica gel; benzene : ethanol = 25 : 4). (Two faster migrating, minor yellow spots were observed in addition, when the diazonium

The yellow products had electronic spectra typical of (*Z*)-pyrromethenones[11] (Fig. 2). Bilirubin IXα contains two different pyrromethenone units, one bearing rings A and B, the other bearing rings C and D. Structures for the two yellow pigments obtained from bilirubin IXα can clearly be assigned from the correlation with the single products obtained from the symmetrically substituted bilirubins IIIα and XIIIα (**1c, b**). The 9-methoxymethylpyrromethenone structures (**2a** for the yellow pigment from **1b**, and **2a, b** from **1a**) were determined from their ^{1}H-NMR and mass spectra. Trimethylsilylation yielded the bis-trimethylsilylated pyrromethenones **3a** and **3b** which were characterized by mass spectrometry. Fragments of the 9-CH_2OCH_3-substituent were observed at M-31 (-OCH_3, 56%) and M-45 (-CH_2OCH_3, 9.5%). As with bilirubin[9,12] silylation occurs both at the carboxyl group and at the lactam (to give the lactim ether). The characteristic ^{1}H-NMR signals of the **2a, b** mixture were at δ 4.45 (s, 2H, $\underline{CH_2}OCH_3$) and at δ 3.27 (s, 3H, $CH_2O\underline{CH_3}$). The remaining NMR and mass spectrometric data accorded with the proposed pyrromethenone structures (**2a, b**).



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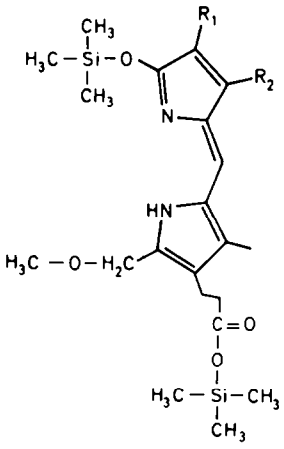

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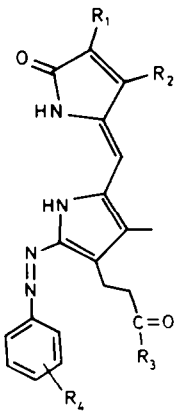


4

R_3 = OH, R_4 = p-SO_3

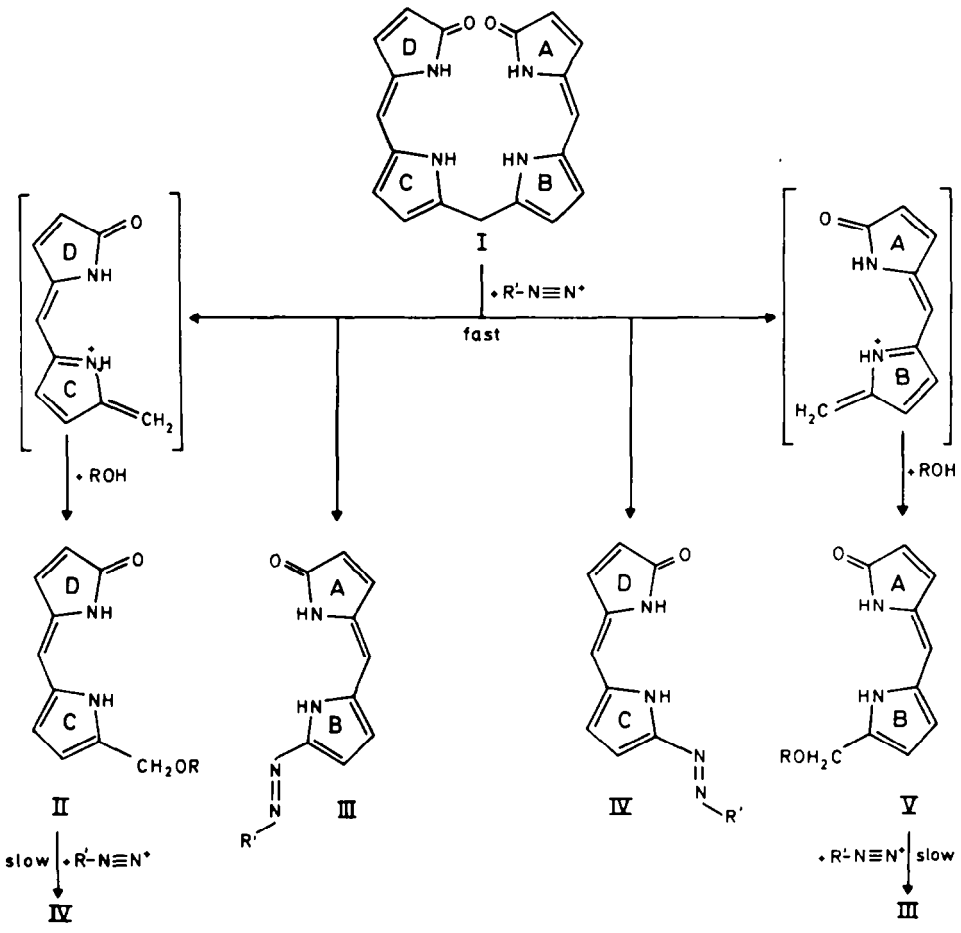
	R_1	R_2
2, 3, 4a:	CH_3	C_2H_3
2, 3, 4b:	C_2H_3	CH_3

Reaction of the yellow products with diazo reagent. The stability of the 9-methoxymethylpyrromethenones (**2a, b**, 47 μM) towards further reaction with the diazonium salts was examined under the same conditions as described for the reaction of the bilirubins in the spectroscopic studies section. In the presence of a 1.5-fold molar excess of diazotized sulfanilic acid, the absorption at 420 nm decreased by 10% and 48% after 1 and 24 hr, respectively, with a concomitant increase around 520 nm by 6 and 20%, corresponding to the formation of azo derivatives. The reaction was more rapid with a 15-fold excess, leading to a decrease of 28, 57 and 69% around 420 nm after 0.25, 1 and 24 hr, respectively, and an increase of 10, 19 and 10% around 520 nm. The half-life of the diazonium salt in methanolic solution has been determined spectrophotometrically (24 hr) from the decrease in absorption at 268 nm. It is apparent from these data, that **2a, b** reacts only slowly with an excess of diazonium salt under the stated reaction conditions. It is also clear, that the 9-azopyrromethenones are not the only products. **2a, b** and **4a, b** have very similar extinction coefficients, but nonetheless the increase at 520 nm amounts to a maximum of 50% of the decrease around 420 nm, corresponding to only 50% conversion of **2a, b** to **4a, b**. Furthermore, the latter absorption is blue-shifted during the reaction from its original position at 420 nm by up to 30 nm (24 hr, 15-fold excess reagent), no isobestic points are observed, and the 520 nm band decreases again upon prolonged reaction with excess reagent. The latter is evidence for one possible side reaction viz. the reaction of the 9-azopyrromethenone **4a, b** with diazonium salts, which has already been observed earlier.[8] The rate of this reaction is much lower than that for bilirubin, but is of the same order of magnitude as that for the 9-methoxymethylpyrromethenone. Hence, the two products **2a, b** and **4a, b** of the diazoreaction of bilirubin are both reactive, and a complete conversion of **1a** into **2a, b** is not observed.

DISCUSSION

The reaction of bilirubin in alcoholic solvents with diazotized aromatic amines leads to a mixture of two principal products. One of them is the well-known 9-azopyrromethenone (III, IV in Scheme 1). It is water soluble when diazotized sulfanilic acid is used as the reagent, and can then easily be isolated from the reaction mixture by solvent partition. It has been characterized by comparison with the electronic spectra recorded in the literature[9] and has not been further examined here. The other product which has hitherto not been characterized has now been identified as a pyrromethenone bearing an alkoxymethyl-substituent at C-9 with the alkoxy substituent derived from the solvent.

Products and/or long-lived intermediates other than the 9-azopyrromethenones have been discussed earlier by several authors. Fischer and Haberland[3] observed that the yield of the 9-azopyrromethenone never exceeded 50% of the theoretical amount, but that 9-unsubstituted pyrromethenones reacted smoothly and completely. They concluded that the first step during the diazo reaction is the hydrolysis of bilirubin into one molecule of a 9-hydroxymethylpyrromethenone and a second molecule of a 9-unsubstituted pyrromethenone. The latter then reacts rapidly with the diazo reagent, whereas the former may condense back slowly to give bilirubin and other products of unknown structure. Overbeek *et al.*[13] have carried out a kinetic study on the diazo reaction of bilirubin. They observed a two-step reaction and suggested the same hydrolytic primary reaction of bilirubin with a rapid attack of the diazonium salt on the pyrromethenone, and a slower but direct—attack on the 9-hydroxymethylpyrromethenone. Treibs and Fritz[14]



Scheme 1.

questioned the hydrolysis of bilirubin as the primary step, and suggested a direct attack of the diazonium salt at either C-9 or C-11 of bilirubin. This view is generally supported by the stability of bilirubin. In particular, the diazo reaction is rapid in acidic as well as in neutral solution. If one takes, by contrast, the scrambling reaction as a measure for a tendency of bilirubin to be cleaved at the central methine bridge, this reaction is very slow at neutral pH.[16]

Further mechanistic work was carried out by Hutchinson and co-workers.[15] They identified for the first time the "missing carbon", i.e. C-10 of bilirubin, as formaldehyde. It was suggested that bilirubin is first attacked at C-9 or C-11 by one molecule of the diazonium salt to yield one molecule of a 9-azopyrromethenone, and one of a dipyrrolic azafulvenium ion as the primary intermediate, which is then stabilized by nucleophilic attack of the solvent (e.g. an alcohol) to yield a 9-alkoxymethylpyrromethenone (Scheme 1). The latter then reacts with a second molecule of the diazonium salt to yield a second molecule of the 9-azopyrromethenone along with formaldehyde. Heirwegh *et al.*[5] also discuss proton abstraction from the azafulvenium ion as a means of stabilizing the primary intermediate.

The results presented here have reconfirmed the sequential mechanism of the diazo reaction (Scheme 1). The practically quantitative yield (93%) of one mole of the 9-methoxymethylpyrromethenone per mole of bilirubin confirms the stabilization of the postulated primary azafulvenium intermediate by nucleophilic attack of the solvent.

The isolation of **2** as intermediates in the diazo reaction provides a basis for further mechanistic and reactivity studies, since the isolated products can be used as substrates in the diazo reaction. Under the reaction conditions employed, we found that the pyrromethenones described above were sufficiently stable towards diazonium ions that they should not be considered as intermediates, but rather as the second product of the reaction besides the 9-azopyrromethenone, with the two products formed in a 1 : 1 molar ratio. We are presently investigating if this is also valid for other conceivable intermediates (e.g. those formed in aqueous medium), and how their reaction with diazonium ions is influenced by the reaction medium.

EXPERIMENTAL

For analytical thin layer chromatography, silica gel plates 10 × 10 cm, HPTLC (Merck, Darmstadt) were used with benzene : absolute ethanol = 25 : 4 as the solvent system. Preparative TLC was done on silica gel H (Merck) plates 20 × 20 cm, 0.5 mm SiO_2. Electronic spectra were recorded with a model 320 spectrophotometer, which allows an automatic base-line correction (Perkin-Elmer, Konstanz). ^{1}H-NMR spectra were recorded in MeOH-d_4 or acetone-d_6 with a model HFX 90 spectrometer (Bruker, Karlsruhe) in the FT mode. Chemical shifts are given as δ (ppm) relative

to $Si(CH_3)_4$ as internal standard. s = singlet, d = doublet, t = triplet, m = multiplet. IR spectra were recorded with a Beckman model IR 33 spectrophotometer. Mass spectra were taken in the EI-mode with a model JMS-D-100 spectrometer (Jeol, Japan) or a model CH4 instrument (Varian-MAT, Bremen). The dipyrrolic pigments were trimethylsilylated prior to mass spectroscopy by dissolving a few crystals of the products in 60 µl pyridine (distilled over KOH) and treatment with 20 µl N,O-bis-(trimethylsilyl)trifluoroacetamide (Pierce) for several hr in a closed vessel. M.p.s are uncorrected. Compound **1a** (biochemical grade, Merck, Darmstadt) was purified by extraction with 0.1 N $NaHCO_3$ and crystallized from $CHCl_3$-MeOH.[16] For the preparation of **1c** and **1b**, **1a** was "scrambled" according to the procedure of McDonagh[16] and separated by preparative TLC.[17] Bilirubin XIIIα was also prepared on a larger scale by the method of Monti and Manitto[10] and crystallized from $CHCl_3$-MeOH. NaOH, $NaNO_2$ and sulfanilic acid were Merck pro analysis reagents.

Spectroscopic studies

The UV-vis spectroscopic studies were generally carried out in MeOH or EtOH containing a trace of DMSO. To prepare this soln, **1a** was first dissolved in a small volume of DMSO, and then diluted with MeOH. The final DMSO concentration was < 1 %. DMSO does not appear to change the reactivity, since qualitatively similar results were obtained in methanolic soln and in pure DMSO.

Preparation of 20 mM diazotized sulfanilic acid reagent

NaOH (1.0 g, 25 mmol) and sulfanilic acid (4.33 g, 25 mmol) were dissolved in *ca* 100 ml glass distilled water. $NaNO_2$ (1.73 g, 25 mmol) was added to the ice-cold soln, which was then made up with water to 250 ml. This soln (20 ml) and 1 N HCl (10 ml) were mixed and kept in an ice bath for 15 min, when water was added to give a final volume of 100 ml. The reagent was stored in a refrigerator. It was not used for longer than one week, and the content of diazotized sulfanilic acid was checked spectrophotometrically at the 268 nm maximum[7] before each experiment. For reactions under neutral conditions, the diazonium salt solution was neutralized with 0.1 N NaOH immediately before addition.

9-Methoxymethylpyrromethenones (**2a, b**)

(a) *As a mixture from bilirubin IXα* (**1a**). Bilirubin IXα (20 mg, 3.42×10^{-5} mol) was dissolved under reflux in $CHCl_3$ (40 ml) and added with vigorous magnetic stirring to MeOH (760 ml). 2.5 ml of a 20 mM soln of diazotized sulfanilic acid reagent (5×10^{-5} mol) were neutralized with 0.1 NaOH to pH 6–7 (final volume about 4 ml) and immediately added. The mixture was stirred for 15 min at ambient temp. The volume of the mixture was reduced to 150 ml in a rotary evaporator. Two phases separated after addition of $CHCl_3$ (150 ml) and water (600 ml) and gentle shaking: a violet aqueous phase and a yellow organic phase. The former was further extracted with $CHCl_3$ (2 × 50 ml). The combined organic phases were washed with water (50 ml) and dried over NaCl. Yields: aqueous phase: 750 ml of $E_{520} = 1.36$, with $\varepsilon_{520} = 29{,}800$[13,19], corresponding to 16.1 mg **4a** + **4b** (95 % of theory); $CHCl_3$ phase: 250 ml of $E_{415} = 3.72$ with $E_{415} = 27{,}800$ in MeOH (see below) corresponding to 10.5 mg **2a** + **2b** (93 % of theory). The $CHCl_3$ soln was evaporated to dryness. The residue was taken up in 2 ml hot CH_2Cl_2 : MeOH (9 : 1) and filtered through cotton wool. n-Hexane was added to the hot soln, until the soln became turbid. After standing at −20° overnight, the crystals (needles) were harvested by centrifugation, washed with 2 ml n-hexane and dried in high vacuum over paraffin at 45° for 12 hr. Yield: 3.2 mg. M.p. slow decomp > 170°. Analytical TLC: Two spots of equal intensity, $R_f = 0.24$ and 0.28. λ_{max} ($\varepsilon \times 10^{-3}$) (MeOH) 420 (27.8), 277 (7.8), 226 (shoulder), 205 nm (18.1). ^{1}H-NMR (CD_3OD) δ 6.73 (m, 2H, vinyl-H_X), 6.31, 6.28 (s, 2H, 5-H), 5.60 (m, 4H, vinyl-$H_{A,B}$), 4.49 (s, $3H^{20}$, 9-CH_2), 2.78 (d, 4H, CH_2CO_2H), 2.51 (d, $3H^{20}$, $CH_2CH_2CO_2H$), 2.24, 2.18, 2.13, 2.02 (s, $12H^{21}$, 2,7- and 3,7-CH_3, respectively, in **2a, b**), the 9^1-OCH_3 signal is hidden under the CH_3OH peak. ^{1}H-NMR (acetone-d_6) δ 9.9 (s, broad, NH), 9.2 (s, broad, NH), 6.83 and 6.43 (m, 1H each vinyl-H_X), 6.22 (s, 2H, 5-H), 5.67 (m, 4H, vinyl-$H_{A,B}$), 4.45 (s, 4H, 9-CH_2), 3.27 (s, 6H, 9-CH_2-O-CH_3), 2.22, 2.15, 2.12, 1.97 (s, 2,7- and 3,7-CH_3, respectively, in **2a,b**), the CH_2–CH_2–CO_2H signals are hidden under a water peak. IR (KBr) ν_{max} 3350, 2970, 2920, 2860, 2810, 1710, 1665, 1630, 1445, 1410, 1370, 1330, 1295, 1260, 1220, 1165, 1080, 980, 920, 860, 740, 665 cm^{-1}. Mass spectrum after trimethylsilylation: *m/e* (relative intensity): 474 (M, 100 %), 459 (−CH_3, 29 %) 446 (−28, 18 %), 443 (−OCH_3, 56 %), 429 (−CH_2OCH_3, 10 %), 427 (−47, 27 %), 357 (11 %), 344 (21 %), 237 (M^{++}, 20 %). M^+ was 100 % with respect to the ions with *m/e* > 150.

(b) *2,7-Dimethyl-3-vinyl-8-carboxyethyl-9-methoxymethylpyrromethenone* (**2a**). The same procedure was used but starting with **1b**. Yield: 61 % in the organic extract after work-up, 13 % after crystallization. M.p. slow decomp > 160°. Analytical TLC: $R_f = 0.24$ (identical on co-chromatography with the slower moving compound isolated from the reaction of bilirubin IXα). λ_{max} ($\varepsilon \times 10^{-3}$) (MeOH) 417 (20.4), 274 (7.4), 227 (shoulder), 206 nm (13.4). ^{1}H-NMR (acetone-d_6) δ 10.0 (s, broad, NH), 9.3 (s, broad, NH), 6.83 (m, 1H, vinyl-H_X), 6.22 (s, 1H, 5-H), 5.66 (m, 2H, vinyl-$H_{A,B}$), 4.46 (s, 2H, 9-CH_2), 3.27 (s, 3H, 9-CH_2OCH_3), 2.12, 1.97 (s, 2,7-CH_3), the $CH_2CH_2CO_2H$ signals are hidden under a water peak. IR (KBr) ν_{max} 3345, 2960, 2910, 2845, 2800, 1705, 1655, 1625, 1440, 1400, 1360, 1320, 1290, 1250, 1210, 1155, 1075, 970, 915, 855, 735, 660 cm^{-1}. Mass spectrum after trimethylsilylation: *m/e* 474 (M^+, 100 %), 459 (−CH_3, 39 %), 446 (−28, 57 %), 443 (−OCH_3, 38 %), 432 (−42, 22 %), 429 (−CH_2OCH_3, 18 %), 427 (14 %), 417 (16 %), 414 (22 %), 400 (12 %), 387 (93 %), 237 (M^{++}, 13 %). M^+ was 100 % with respect to the ions with *m/e* > 150.

(c) *3,7-Dimethyl-2-vinyl-8-carboxyethyl-9-methoxymethylpyrromethenone* (**2b**). This was prepared on an analytical scale from **1c** (0.15 mg) by essentially the same method as described for **2a**. Analytical TLC: $R_f = 0.28$ (upon co-chromatography identical to the faster moving isomer obtained from **1a**). λ_{max} (MeOH): 418, 276 nm.

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[20]Theoretically 4H: possible $^1H/^2H$-exchange.
[21]Unequal intensities of some of the signals indicate that the two isomers are not present in a 1 : 1 mixture.