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Synthesis of water-soluble bile pigments bound to amine-ended monomethoxypolyethyleneglycol: thiol addition and attempted enzymatic reduction of a bilindione derivative*

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Abstract. The water-soluble amide to an NH_2-ended monomethoxypolyethyleneglycol (MPEG-NH_2, molecular mass of about 2000) of the dipyrrinone xanthobilirubic acid (XBR, **1**) and the bis-amides of mesobiliverdin-XIIIα (MBV, **2**) and mesobilirubin-XIIIα (MBR, **3**) have been prepared with high yields. Contrary to what is observed with biliverdin-IXα, **4**, the enzymatic reduction of the mesobiliverdin derivative **2-MPEGA** to the corresponding mesobilirubin **3-MPEGA** by the soluble biliverdin reductase/NADPH system in pH 7.4 aqueous phosphate does not occur. In contrast, thiol addition to **2-MPEGA** and to **4** under similar conditions is immediate, although this equilibrium is slightly less favourable for **2-MPEGA**. These results enable us to discount the intrinsically low reactivity of **2-MPEGA** towards thiols as the reason for its lack of enzymatic reduction, and suggest instead that this particular mesobiliverdin cannot fit properly into the enzyme binding site, either because of steric hindrance or the lack of the two propionic acid groups.
Key words. Polyethyleneglycol amide; water-soluble polymer; biliverdin; bilirubin; UV-visible kinetics; thiol addition; enzymatic reduction; biliverdin reductase.

Water-soluble bilirubin derivatives are important for in vitro and in vivo studies of metabolism and excretion as well as structural and spectroscopic investigations [1, 2] in aqueous media at physiological pH, as the number of bilirubin derivatives truly soluble under these conditions is low. Among them are the natural glucuronides [2] and the related, commercially available taurates, in addition to preparations such as complexes with albumins and other proteins [3] and surfactants [4]. In the past, we have reported a number of polyethyleneglycol-based water soluble derivatives of both linear and cyclic pyrrole pigments, including esters of xanthobilirubic acid (XBR, **1**) [5], the thioether adduct to the exo-vinyl group of bilirubin-IXα, and the monoester and the monoamide of mesoporphyrin-IX monomethyl ester. For linear and cyclic pyrrole pigments containing two carboxylic acid groups, such as bilirubin-IXα, biliverdin-IXα and mesoporphyrin-IX, all attempts to prepare the bis-derivatives at both carboxylic acid groups (either as esters or amides) were unsuccessful, and difficult to separate mixtures of products were obtained, presumably as the result of the steric hindrance imposed by the first polymeric chain introduced. Also related to the derivatives of the present work are the polyacrylamide-bound violinoid and 2,3-dihydrobilindione systems recently reported by Falk et al. [6].

In this paper, we extend our own synthetic procedure toward the water-soluble xanthobilirubic acid MPEG ester, **1-MPEGE** [5], to the preparation of xanthobilirubic acid MPEG amide, **1-MPEGA** (formula scheme), and use well-known reactions of bile-pigment chemistry [5, 7, 8] to prepare the mesobiliverdin and mesobilirubin derivatives **2-MPEGA** and **3-MPEGA**.

The aqueous solubility of the verdin **2-MPEGA** makes it an adequate substrate for the study of the reduction step of the biliverdin reductase system [9]. Frydman et al. studied the substrate specificity and kinetics by this enzyme [10]. After examining a series of synthetic biliverdins with a varying number of propionate and acetate chains, they found that the presence of at least two propionates in the biliverdin structure was needed for substrate activity. Substitution of one or two propionates by acetate led to loss of recognition by the enzyme. The last observation suggests to us that the important property for substrate specificity may be not biliverdin acidity but rather its ability to hydrogen bond to particular sites of the enzyme. In agreement with this interpretation is the report by O'Carra and Colleran [11] that esterification of only one of the two propionates decreases substrate effectiveness only slightly,

* For reference see: Fontich M., Fors P., Sesé M. L. and Trull F. R. (1994) Polymer bound pyrrole compounds. VIII. Water-soluble pyrrole pigments carrying polyether side chains. Eur. Polym. J. **10:** 1143-1149.

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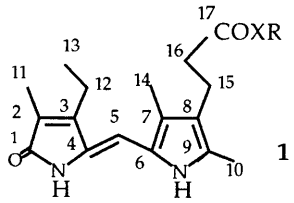

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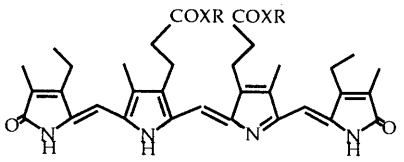

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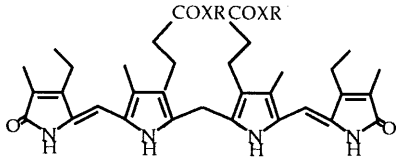

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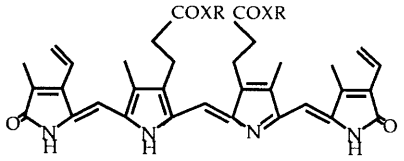

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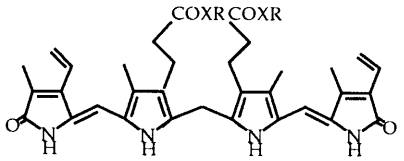

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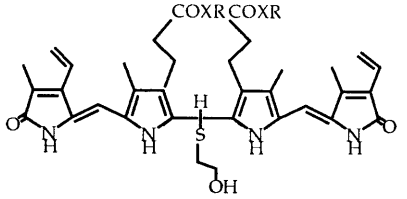

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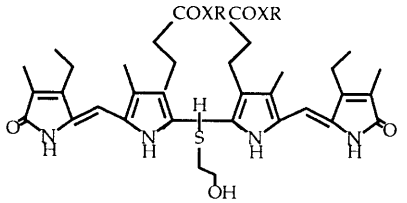

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Compound	XR
1 to **7**	OH
1-MPEGA to **7-MPEGA**	NH-MPEG
1-MPEGE	O-MPEG

MPEG stands for $CH_3O(CH_2CH_2O)_{44}$-CH_2CH_2-

whereas esterification of both abolishes substrate effectiveness entirely. In this respect, the results of enzyme recognition of the bis-amide derivative **2-MPEGA**, with two –C(O)–NH– groups capable of participating efficiently in the formation of hydrogen bonds, will help establish whether it is hydrogen bonding that is important for recognition. Eventually, the mono conjugate should also be a good model to check substrate specificity of BV-reductase further.

Frydman's group also demonstrated that the addition of one biliverdin reductase thiol group to biliverdin is the initial step in the reaction sequence leading to bilirubin [12]. Apparently, this covalent binding of the biliverdin to the active centre provides a proper orientation for the following reduction step using the biological hydride donors NADH or NADPH. In a further step, Falk and Marko studied the reduction of biliverdin dimethyl ester and its 10-thioethanol addition product by LiBH4 in d_6-DMSO, and found similar reaction velocities for the two substrates [13]. From this they concluded that the addition of the enzyme thiol group to C10 of biliverdin is not intended to accelerate the velocity of the reduction step. We therefore found it of interest to investigate (1) whether the mesobiliverdin derivative **2-MPEGA** can be enzymatically reduced in vitro to the corresponding mesobilirubin **1-MPEGA** by the soluble biliverdin reductase/NADPH system in pH 7.4 aqueous phosphate and, if so, what is the rate of this process relative to that of reduction of biliverdin-IXα, **4**, to bilirubin-IXα, **5**; and (2) whether the mesobiliverdin derivative **2-MPEGA** undergoes thiol addition under similar conditions and, if so, whether the relative rates of this process and of thiol addition to **4** correlate with the relative rates of enzymatic reduction of the same two compounds.

Materials and methods

Chemicals

Bilirubin-IXα, 5, and DDQ (98%) (Janssen Chimica), polyethylene glycol monomethyl ether (MPEG-OH) of $M_n = 2000$ and 2-mercaptoethanol (Fluka), TFA and $NaBH_4$ (Merck), DCC and DMAP (Aldrich), and fatty acid free human serum albumin (HSA) and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) (Sigma), were all used without further purification. Anhydrous THF was obtained from the solvent grade product (Normasolv, Scharlau, Barcelona, Spain) by drying upon and distilling from calcium hydride (Aldrich) first and lithium aluminum hydride (Aldrich) thereafter. Triethylamine (Merck) was kept on potassium hydroxide and distilled from this agent shortly before use. Argon-saturated, deionized water was used in the thiol addition and enzymatic reduction studies. All other solvents were rendered oxygen-free, argon saturated by several cycles of degassing under vacuum at room temperature under magnetic stirring followed

by argon saturation. The synthesis of the following compounds is described in the literature: xanthobilirubic acid, **1** [14], biliverdin-IXα, **4** [8, 15], polyethylene glycol mono-methyl ether amino derivative (MPEG-NH_2) [16].

The cytosolic fraction from rat liver was obtained according to the following procedure: immediately after the sacrifice of the rats, the liver was extracted and homogenized in 3 vol (w/v) of 0.25 mol dm^{-3} sucrose in a Potter-Elvejhem apparatus. The homogenate obtained was centrifuged at 10,000 × g for 10 min, and the resulting supernatant was centrifuged at 105.000 × g for 60 min. All the steps were carried out at 277 K. Reactions were typically carried out in the dark and under argon.

Analytical methods

Analytical thin layer chromatography (TLC) was carried out on Merck 60 F_{254} silica gel plates (0.2 mm layer). High performance liquid chromatography (HPLC) was carried out in a Waters Associates instrument equipped with two pumps, using a C18 column (Radial Pack) and 0.1 mol dm^{-3} di-n-octylamine acetate in MeOH as mobile phase [17]; the flow rate was 1 $cm^3 \cdot min^{-1}$. Melting points (not corrected) were determined on a Kofler-Reichert micro-hot stage apparatus. UV-visible (UV-VIS) spectra were recorded on a Varian Cary 5E instrument. ^{1}H- and ^{13}C-NMR spectra were run on a Varian Gemini XL-200 (200.6 MHz) or a Varian Unity-300 (300 MHz) spectrometer using tetramethylsilane (TMS) as internal standard in deuteriochloroform (99.9% d_1). Immediately before sample preparation, this solvent was filtered through a short column of basic Al_2O_3 (Act. I, from Merck) and the first few drops discarded. IR spectra were determined in KBr disks with a Fourier-Transform Nicolet spectrophotometer. Mass spectra (MALDI) were run on a KRATOS instrument equipped with a 20 keV N_2 laser and for TOF analysis, using 10 pmol of product in 0.5 mm^3 of α-cyano-4-hydroxy-cinnamic acid (matrix) and 100 shots. The instrument was calibrated with the commercial MPEG-OH ($M_n = 2000$) used in the synthesis. For all compounds, a gaussian distribution of peaks differing from each other by 43 mass units (i.e. ethylene glycol units) and centred near the expected molecular mass was obtained. Except where otherwise indicated, all spectroscopic measurements were performed at room temperature.

Xanthobilirubic acid monomethoxy polyethylene glycol amide (1-MPEGA)

A modification of the procedure previously reported for the preparation of xanthobilirubic acid monomethoxy polyethylene glycol ester (**1-MPEGE**) [5] was used.

A yellow-greenish solid (90% yield based on MPEG-NH_2; one spot by TLC (SiO_2; CH_2Cl_2: MeOH; 10: 1; $R_f = 0.35$; Al_2O_3; CH_2Cl_2: MeOH; 10: 1; $R_f = 0.7$) and by HPLC ($R_t = 5.5$ min) was obtained which was spectroscopically identified as the title compound. M.p. 50–52 °C (with decomposition). ^{1}H-NMR (300 MHz, δ, $CDCl_3$): 10.4 (broad s, 1H, pyrrole NH), 10.0 (sharper broad s, 1H, lactam NH), 6.2 (broad s, 1H, amide NH), 6.05 (s, 1H, =CH–), 3.6 (s, ca. 180H, polymeric -CH_2-), 3.3 (s, 3H, polymeric –O–CH_3), 2.7 (t, 2H, –(CH_2–CH_2–COO–), 2.5 (q, 2H, –CH_2–CH_3), 2.3 (t + s, 2H + 3H, –CH_2–CH_2–COO–, C3′– or C5′–CH_3), 2.1 (s, 3H, C5′– or C3′–CH_3), 1.9 (s, 3H, C3–CH_3), 1.2 (t, 3H, –CH_2–CH_3). ^{13}C-NMR (300 MHz, δ, $CDCl_3$): 173.7(C1), 172.6 (C17), 148.0 (C3), 131.2 (C9), 127.4 (C4), 124.0 (C2), 122.7 (C6), 122.3 (C8), 119.3 (C7), 100.3 (C5), 71.8–69.8 (polymer), 58.9 (–OCH_3), 39.2, 33.9 (unidentified signals), 37.5 (C16), 20.4 (C15), 17.8 (C12), 14.9 (C13), 11.5 (C14), 9.5 (C11), 8.4 (C10); assignment based on that in ref. 14 for XBR-NHMe amide. UV-VIS (CH_2Cl_2): $\lambda_{max}(\varepsilon) = 407$ nm (24,800); (H_2O): 412 (26,000). IR (KBr):$\nu = 3360$ (NH), 2890 (MPEG), 1670 (amide + lactam C=O), 1635 (C=C), 1470, 1345, 1285, 1245, 1150, 1110 (MPEG), 1065, 970, 850 cm^{-1}. MS (MALDI-TOF): m/e (%) ≈ 2300.

Mesobiliverdin-XIIIα bis monomethoxy polyethylene glycol amide (2-MPEGA)

To a solution of xanthobilirubic acid derivative **1-MPEGA** (115 mg, 0.05 mmol) in absolute THF (35 cm^3) were added TFA (0.25 cm^3) at once followed by a solution of DDQ (15 mg, 0.06 mmol) in absolute THF (2.5 cm^3) dropwise over 30 min at 293 K and under magnetic stirring. The blue-green solution was cooled in an ice bath, triethylamine was added up to neutral pH (0.2 cm^3), and 3-fold excess cold ether was added to precipitate the biliverdin derivative **2-MPEGA**. The precipitate was washed repeatedly with cold ether and the residue was dried.

A mixture was obtained (by TLC) consisting mostly of a blue product (the title biliverdin) contaminated by some yellow impurity. The verdin was purified by preparative TLC (Al_2O_3; CH_2Cl_2: MeOH; 10: 1) and spectroscopically identified as the title compound (80% yield; one spot by TLC (SiO_2; CH_2Cl_2: MeOH; 10: 1; $R_f = 0.05$; Al_2O_3; CH_2Cl_2: MeOH; 10: 1; $R_f = 0.8$). M.p. 45–47°C. ^{1}H-NMR (300 MHz, δ, $CDCl_3$): 7.6 (2xt, 2H, amide NH), 6.9 (s, 1H, =C10H–), 5.9 (s, 2H, =C5,15H–), 3.6 (s, ca. 360H, polymeric –CH_2–), 3.4 (s, 6H, polymeric –O–CH_3), 2.9 (t, 4H, –CH_2–CH_2–COO–), 2.5 (q, 4H, –CH_2–CH_3), 2.4 (t, 4H, –CH_2–CH_2–COO–), 2.1 (s, 6H, C7,13–CH_3), 1.8 (s, 6H, C2,18–CH_3), 1.2 (t, 6H, –CH_2–CH_3). ^{13}C-NMR (300 MHz, δ, $CDCl_3$): 172 (lactam and amide C=O), 146.5 (C3, C17), 140.5, 139.5, 138, 131, 127, 126.5, 125 (rest of lactam and pyrrole α,β-carbons), 96 (C5,15), 95 (C10), 71.8–70.0 (polymer –OCH_2CH_2O–), 58.9 (poly-

mer –OCH_3), 39.2, 32, 26 (unidentified signals), 38 (–CH_2CH_2CO–), 22.6 (–CH_2CH_2CO–), 17.7 (–CH_2CH_3), 14.4 (–CH_2CH_3), 14 (C7,13–CH_3), 8.3 (C2,18–CH_3). UV-VIS (CH_2Cl_2): $\lambda_{max}(\varepsilon) = 370$ nm (35,800), 626 (9,800); (H_2O): 367 nm (35,600), 650 (11,000). IR (KBr):$\nu = 3423$ (NH), 2890 (MPEG), 1700 (amide + lactam C=O), 1656 (C=C), 1113 (MPEG) cm^{-1}. MS (MALDI-TOF): m/e (%) ≈ 4500.

Mesobilirubin-XIIIα bis monomethoxy polyethylene glycol amide (3-MPEGA)

Dry MeOH (1 cm^3) was added with rapid stirring to a mixture of the mesobiliverdin derivative **2-MPEGA** (30 mg, 6.5 mmol) and sodium borohydride (38 mg, 1 mmol) cooled in an ice bath. Since a greenish colour persisted after 15 min, the mixture was allowed to come to room temperature and to react for a further 15 min, when the colour was completely yellow. While argon was bubbled through the mixture, 0.1 mol dm^{-3} aqueous HCl was added until neutral pH was reached. The mixture was extracted with dichloromethane (3×5 cm^3), the organic extract washed with water and filtered through paper to yield the title rubin as a yellow solid (80% yield; one spot by TLC (SiO_2; CH_2Cl_2: MeOH; 10: 1; $R_f = 0.1$; Al_2O_3; CH_2Cl_2: MeOH; 10: 1; $R_f = 0.6$) which was spectroscopically identified as the title compound. M.p. 48–49 °C (with decomposition). ^{1}H-NMR (300 MHz, δ, $CDCl_3$): 10.83 (sharper broad s, 2H, lactam NH), 10.26 (broad s, 2H, pyrrole NH), 9.01 (broad s, 2H, amide NH), 5.95 (s, 2H, =C5,15H–), 4.03 (s, 2H, –C10H_2–), 3.6 (s, ca. 180H, polymeric –CH_2–), 3.37 (s, 6H, polymeric –O–CH_3), 2.82 (t, 4H, –(CH_2–CH_2-COO–), 2.47 (q, 2H, –CH_2–CH_3), 2.2 (s, 6H, C7,13–CH_3), 2.1 (t, 4H, –CH_2–CH_2–COO–), 1.84 (s, 6H, C2,18–CH_3), 1.1 (t, 6H, –CH_2–CH_3). UV-VIS (CH_2Cl_2): $\lambda_{max}(\varepsilon) = 419$ (31,200), 400 nm (sh, 30,000); (H_2O): 420 (27,300), 394 (sh, 25,300). IR (KBr):$\nu = 3430$ (NH), 2888 (MPEG), 1685 (amide + lactam C=O), 1640 (C=C), 1115 (MPEG) cm^{-1}. MS (MALDI-TOF): m/e (%) ≈ 4800.

Reduction by the biliverdin reductase system of the biliverdin-IXα-HSA 1:1 complex, 4-HSA, and of mesobiliverdin-XIIIα bis monomethoxy polyethylene glycol amide, 2-MPEGA

The procedure used is a modification of the one previously reported [11, 18, 19]. Reduction of each biliverdin was monitored by UV-visible spectroscopy using 1 cm path-length cuvettes thermostatized at 310 K. First, a series of blank assays was carried out. This confirmed that no reaction occurs between each biliverdin ($\lambda_{max} =$ 387, $\varepsilon = 40{,}000$, 670 nm, $\varepsilon = 13{,}600$ for 4-HSA; 367, $\varepsilon = 35{,}600$, 650 nm, $\varepsilon = 11{,}000$ for **2-MPEGA**) and either the homogenate ($\lambda_{max} = 415$ nm) or NADPH ($\lambda_{max} = 339$ nm, $\varepsilon = 6{,}200$). In contrast, NADPH was found to be oxidized to NADP ($\lambda_{max} = 260$ nm, $\varepsilon =$ 18,000) both by the aqueous buffer (ca. 10% oxidation after 1 h) and by the homogenate (ca. 40% oxidation after 1 h), the last reaction rate being comparable to that for the enzymatic reduction of biliverdin-IXα (below). In the enzymatic reduction, the complete reaction mixture was contained in a final volume of 3 cm^3: 1 cm^3 of NADPH aqueous solution in 0.1 mol dm^{-3} sodium phosphate buffer, pH 7.4, to a final concentration 90 mmol dm^{-3}; 1.9 cm^3 of verdin solution (**4**-HSA [20] or **2-MPEGA**) in the same buffer, to a final concentration 20 mmol dm^{-3}. In the reference cell, the biliverdin was replaced by buffer. Spectra from 300 to 750 nm were monitored for approximately 1 h, starting when 0.1 cm^3 of the rat liver cytosolic fraction were added to the two cuvettes. After ca. 1 h the isosbestic points began to disappear, indicating that additional processes were competing. The formation with time of the bilirubin-IXα-HSA complex, **5**-HSA, and eventually of the mesobilirubin-XIIIα derivative, **3-MPEGA**, was calculated from the decrease in optical density at the maximum absorption wavelengths of the verdin used (best seen from the ca. 660 nm band). Eventually, the formation of the relevant bilirubin can be estimated from the increase in optical density at the maximum absorption wavelength (i.e. 454 nm and 407 nm respectively). An additional series of experiments was carried out comparing the evolution with time of the mixture of verdin, homogenate and NADPH (sample cuvette) to that of verdin plus homogenate (no NADPH; reference cuvette). In this case, approximately the same verdin reduction rates as above were obtained when the low energy band of the verdin was monitored; however, the contribution by NADPH oxidation (calculated independently) had to be subtracted when the decrease of the high energy band of the verdin was used to estimate reduction rates.

Thiol addition to biliverdin-IXα, 4, and to mesobiliverdin-XIIIα bis monomethoxy polyethylene glycol amide, 2-MPEGA

These reactions were monitored by UV-visible absorption spectroscopy using 1 cm path-length cuvettes thermostatized at either 293 K or 310 K. A ca. 36 μmol dm^{-3} verdin (**4** or **2-MPEGA**) solution in 0.1 mol dm^{-3} aqueous sodium phosphate buffer, pH 7.4, was used. For biliverdin-IXα, **4**, DMSO was used to a final 2% (v:v) to get all the pigment in the aqueous buffer solution. In one series of experiments, the reference cell contained aqueous phosphate; in the second one, the two cells contained the same biliverdin solution (above). In both cases, the reaction was initiated by addition of increasing excesses (in the range of about 10 to about 100 mmol dm^{-3}) of 2-mercaptoethanol to the sample cuvette (in the first series, also to the reference cuvette).

In the first series of experiments, starting with both verdins and at both temperatures, immediately after each thiol addition a red shift of the Soret absorption maximum (originally at 376 nm for **4**; 367 nm for **2-MPEGA**), and a decrease in the size of the Q-band (ca. 660 nm for **4**; ca. 650 for **2-MPEGA**) were observed. These correspond to formation of increasing percentages of the thiol adduct ($\lambda_{max} = 401$ nm for **6**; 387 nm for **7-MPEGA**). From the absorption maximum of each mixture and those of the pure verdin and thiol adduct, the molar fraction in each component was established. The same information was obtained from the decrease, relative to its initial value, of the absorption of the Q-band. With both verdins, the spectra of the mixtures containing higher thiol excesses needed to be recorded immediately after thiol addition, since a secondary process (decomposition of the thiol adduct? [12]) occurs which leads to a decrease in the size and a simultaneous red shift of the maximum of the thiol adduct. In the second series of experiments, thiol addition resulted in the growth of negative peaks near the absorption maxima of the verdins (ca. 360 and 660 nm), and of positive peaks near the absorption maxima of the corresponding thiol adducts (ca. 400 nm). From these, and the initial absorbances of each sample, the composition of each mixture was obtained. The spectra of the mixtures containing higher thiol excesses were recorded immediately after thiol addition.

Results and discussion

Synthesis and characterization of polymer-bound pigments

Xanthobilirubic acid, **1**, was obtained by total synthesis as previously reported [14]. The monomethoxypolyethyleneglycol amide of xanthobilirubic acid, **1-MPEGA**, was prepared by reaction of the free acid, **1**, with monomethoxypolyethyleneglycol amine, MPEG-NH_2, [16] using dicyclohexylcarbodiimide/dimethylaminopyridine (DCC/DMAP) as the coupling reagent in dry dichloromethane [5]. Due to the difficulty in separating **1-MPEGA** from unreacted MPEG-NH_2, and in order to favour the conversion of all MPEG-NH_2, an approximate 10% molar excess of **1** was used. In spite of this precaution, the isolated amide, **1-MPEGA**, contained approximately 30% MPEG-NH_2, as deduced from the ε value in the UV-VIS spectrum of the reaction product in CH_2Cl_2, and assuming for the pure amide the literature value [14] for xanthobilirubic acid methyl amide.

The amide **1-MPEGA** was identified spectroscopically: in the IR, the amide C=O band near 1670 cm^{-1} overlaps the chromophore lactam C=O band; in the UV-VIS, the spectrum in water ($\lambda_{max} = 412$ nm) is similar to that in CH_2Cl_2 ($\lambda_{max} = 407$ nm) with a slight broadening of the absorption band in water, perhaps due to some aggregation. In addition, these maxima are similar to those of xanthobilirubic acid methyl amide respectively in MeOH and $CHCl_3$, suggesting that in **1-MPEGA** the conformation of the chromophore is not significantly altered by the polymeric chain. However, the similarity with XBR methyl amide breaks down in the ^{1}H-NMR in $CDCl_3$, where **1-MPEGA** presents two NH singlets near 10.4 (broader, pyrrole?) and 10.0 ppm (sharper, lactam?). This behaviour does not parallel that of any previously reported dipyrrinone. Indeed, in intermolecularly hydrogen-bonded dipyrrinones a planar dimeric form is characterized by a sharper lactam NH near 11.2 ppm and a broader pyrrole NH near 10.3 ppm [21–24]. Small deviations from this behaviour have not been considered significant and a planar dimeric form has been proposed for them as well. This is the case for the model XBR methyl amide where the lactam and pyrrole NHs (obtained by extrapolation from the spectra in $CDCl_3$-DMSO-d_6 mixtures) have been reported to appear at 10.9 and 10.1 ppm respectively [14]. It is also the case for XBR MPEG ester, in which the NHs appear at 10.8 and 10.2 ppm [5]. More recently, a new type of dimer has been reported for dipyrrinone acids involving carboxylic acid to dipyrrinone intermolecular hydrogen bonds [25]. These dimers are characterized by the pyrrole NH appearing at 1–1.4 ppm higher fields relative to the traditional planar dimers; the lactam NH is also shifted to higher fields but the intensity of this shift depends on the nature of the alkanoic acid chain substituting position C(8) of the dipyrrinone. For propionic acid (i.e. XBR), this shift is ca. 0.6 ppm and the experimental δ values for this dipyrrinone are 10.60 and 8.84 ppm. The amide NH of **1-MPEGA** appears as a multiplet (two triplets of *syn* and *anti* –C(O)–NH conformers) near 6.2 ppm, a value similar to the one reported for the amide NH of XBR α-methylbenzylamide in $CDCl_3$ [26]. In summary, none of the reported XBR derivatives are good models for **1-MPEGA**; for this compound in chloroform an essentially dimeric structure, with less eff ective hydrogen bonding (relative to XBR methyl amide and other low molecular weight secondary amides) involving the lactam and pyrrole NHs, but not the amide NH, can be postulated.

The monomethoxypolyethyleneglycol bis-amide of mesobiliverdin XIII-α, **2-MPEGA**, was prepared with excellent yield from the dipyrrinone **1-MPEGA** using the 2,3-dichloro-5,6-dicyano-1,4-benzoquinone/trifluoroacetic acid (DDQ/TFA)-catalyzed self-condensation of dipyrrinones to biliverdins [7]. The product was identified spectroscopically: the UV-VIS spectra in water [$\lambda_{max} = 367$, 650 (very broad) nm] and in CH_2Cl_2 [$\lambda_{max} =$ 370, 626 nm] are similar, and do not differ significantly from the absorption spectra of other mesobiliverdins in MeOH. The ^{1}H-NMR and ^{13}C-NMR spectra in $CDCl_3$ are also similar to those of MBV-XIIIα except for the polymer and amide signals.

The monomethoxypolyethyleneglycol bis–amide of mesobilirubin XIII-α, **3-MPEGA**, was prepared with good yield from the corresponding verdin, **2-MPEGA**, using the $NaBH_4$/MeOH reduction of biliverdins to bilirubins [8]. The process was significantly slower than described for the reduction of biliverdin-IXα and required room temperature, a result that points to the hindering effect of the two polymeric chains towards the approach of the reducing agent to the central methine carbon (C10) of the verdin.

The rubin was identified spectroscopically; the UV-VIS spectra in water ($\lambda_{max} = 420$, shoulder near 394 nm) and in CH_2Cl_2 ($\lambda_{max} = 419$, shoulder near 400 nm) are similar except for some band broadening in water; more interestingly, they are noticeably blue-shifted when compared to other mesobilirubins (MBR-XIIIα in MeOH has $\lambda_{max} = 426$, shoulder near 401 nm [27]). This difference in absorption characteristics can be interpreted in terms of the exciton coupling model [28–32]. In this model, the slight shift in the absorption of mesobilirubin **3-MPEGA** relative to the component dipyrrinone, **1-MPEGA** indicates a small interchromophoric orbital overlap, characteristic of a folded conformation (ca. 100° interplanar angle) [33]. In the ^{1}H-NMR in $CDCl_3$, two broad singlets near 10.83 and 10.26 ppm are assigned to the lactam and pyrrole NHs respectively. The amide NH appears as a multiplet near 9.01 ppm. These values are reminiscent of those reported for bilirubin-IXα mono- and bis-2-butyl amide [34] for which a folded conformation stabilized through propionamide NH- (rather than propionic acid OH-) mediated intramolecular hydrogen bonding. Additional, much smaller broad singlets near 10.88, 10.32 and 9.56 ppm may be tentatively attributed to some contamination by MBR-XIIIα mono-MPEG amide (from TFA-catalyzed partial hydrolysis of **2-MPEGA**?). All this evidence points to an essentially folded conformation for **3-MPEGA**, as reported for bilirubin and its 2-butyl amides; the steric hindrance by the two high molecular weight side chains must result in a slight modification of this conformation so as to accommodate the polymeric chains.

Enzymatic reduction [9, 12]

Each of the biliverdins under study [i.e., biliverdin-IXα, **4**, as its 1:1 complex with human serum albumin (HSA), and the mesobiliverdin derivative **2-MPEGA**] in pH 7.4 aqueous buffer was incubated aerobically at 310 K with a $105{,}000 \times g$ supernatant fraction of rat liver homogenate in the presence of NADPH, and the formation of the corresponding bilirubin (i.e. respectively **5**-HSA and **3-MPEGA**) was determined from the increase in optical density at the maximum absorption wavelength in the system used (i.e. 454 nm and 417 nm respectively).

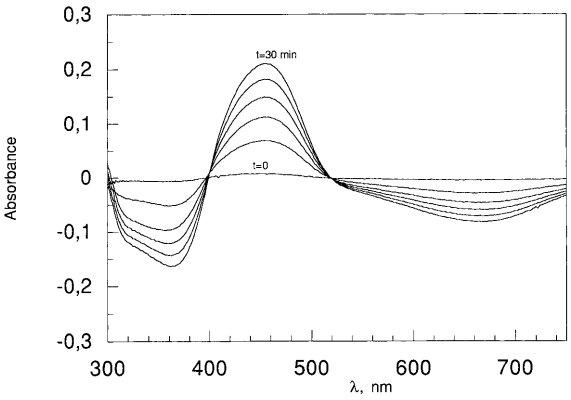

Figure 1. UV-visible spectra for the reduction of the biliverdin-IXα-human serum albumin complex, **4**-HSA, by the biliverdin reductase system (from the cytosolic fraction of rat liver homogenate) in 0.1 mol dm^{-3} aqueous sodium phosphate buffer, pH 7.4, at 310 K. Reference cell (1 cm path): NADPH solution (2.9 cm^3; final concentration 90 μmol dm^{-3}) in buffer, cytosolic fraction in 0.25 mol dm^{-3} sucrose (0.1 cm^3; see 'Materials and methods'). Sample cell: biliverdin-HSA solution (1.9 cm^3; final concentration 20 μmol dm^{-3}) and NADPH solution (1.0 cm^3; final concentration 90 μmol dm^{-3}), both in buffer, rat liver homogenate in 0.25 mol dm^{-3} sucrose (0.1 cm^3; see 'Materials and methods'). Spectra recorded every 6 min, from t = 0 min to t = 30 min. The negative peaks near 350 and 650 nm correspond to biliverdin reduction; the positive peak near 450 corresponds to bilirubin formation. After 30 min, the reduction in absorbance at 370, 660 nm is about 30% relative to biliverdin absorbance at t = 0.

Figure 1 shows the evolution with time of the difference spectra corresponding to the enzymatic reduction of the biliverdin-IXα-HSA complex, **4**-HSA. The disappearance of **4** can be monitored from the negative peaks near 370 and 660 nm, while the concomitant formation of the bilirubin-IXα-HSA complex, **5**-HSA, accounts for the positive peak near 454 nm. Under these conditions (see 'Materials and methods'), about 30% of the starting biliverdin is reduced after 30 min.

The evolution with time of the difference spectra corresponding to the biliverdin reductase/NADPH system using the mesobiliverdin-XIIIα MPEGA, **2-MPEGA**, as substrate is shown in figure 2. No reduction is seen in this case. The absorption maxima of the verdin 'Soret' band (near 367 nm) and of the expected reduction product, i.e. the mesobilirubin-XIIIα MPEGA, **3-MPEGA** (ca. 417 nm) are much closer to each other (50 nm vs 84 nm for reduction of **4**-HSA to **5**-HSA) and this renders monitoring the process difficult. In spite of this, it seems clear that **2-MPEGA** does not disappear (even after several hours, the difference spectra look the same), and we attribute the small negative peak observed near 340 nm to oxidation of some NADPH by an impurity contaminating **2-MPEGA**. The absence of a negative peak near 650 nm (Q band of **2-MPEGA**) confirms that, under these conditions, the mesobiliverdin MPEGA is not reduced.

In order to discount an essential role by the HSA in the distinct behaviour towards enzymatic reduction of the

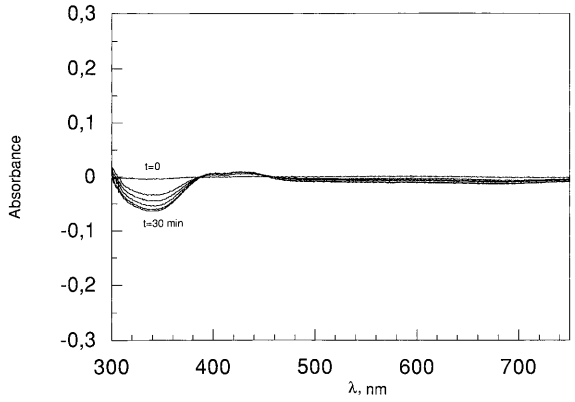

Figure 2. UV-visible spectra for the reduction of mesobiliverdin-XIIIα-MPEG amide, **2-MPEGA**, by the biliverdin reductase system (from the cytosolic fraction of rat liver homogenate) in 0.1 mol dm^{-3} aqueous sodium phosphate buffer, pH 7.4, at 310 K. Reference cell (1 cm path): NADPH solution (2.9 cm^3; final concentration 90 μmol dm^{-3}) in buffer, cytosolic fraction in 0.25 mol dm^{-3} sucrose (0.1 cm^3; see 'Materials and methods'). Sample cell: **2-MPEGA** solution (1.9 cm^3; final concentration 20 μmol dm^{-3}) and NADPH solution (1.0 cm^3; final concentration 90 μmol dm^{-3}), both in buffer, rat liver homogenate in 0.25 mol dm^{-3} sucrose (0.1 cm^3; see 'Materials and methods'). Spectra recorded every 6 min, from t = 0 min to t = 30 min. No increase with time is observed of a negative peak near 650 nm (i.e. no mesobiliverdin disappearance) and of a positive peak near 450 nm (i.e. no mesobilirubin formation).

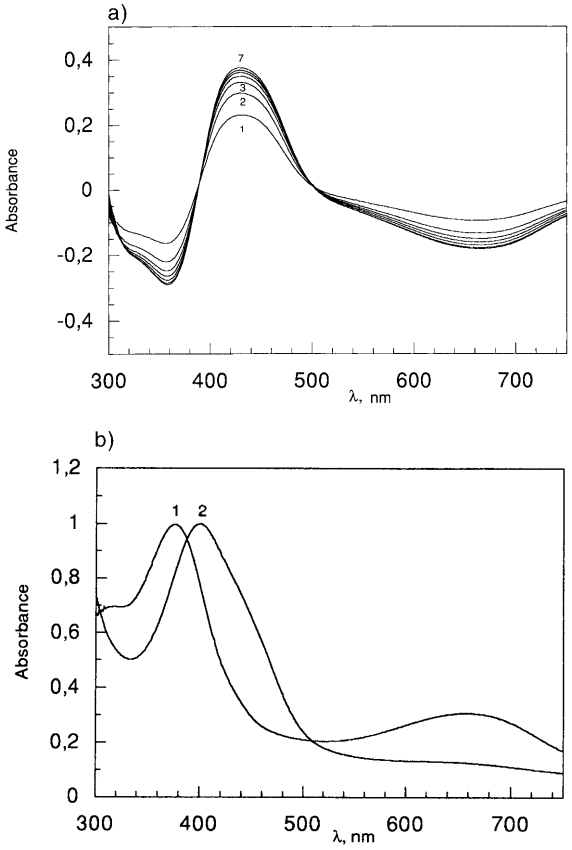

Figure 3. (*a*) UV-visible spectra of the reaction of biliverdin-IXα, **4**, with 2-mercaptoethanol in 0.05 mol dm^{-3} aqueous sodium phosphate buffer, pH 7.4, at 293 K. Reference cell (1 cm path): biliverdin solution 36 μmol dm^{-3}, in buffer containing 2% (v:v) DMSO. Sample cell: reference solution plus 2-mercaptoethanol. (1) 10 mmol dm^{-3}, (2) 20 mmol dm^{-3}, (3) 30 mmol dm^{-3}, (4) 40 mmol dm^{-3}, (5) 50 mmol dm^{-3}, (6) 75 mmol dm^{-3}, (7) 100 mmol dm^{-3}. (*b*) UV-visible spectra vs buffer solution of (1) sample cell before the addition of thiol (i.e. **4**) and (2) sample cell after the addition of 2-mercaptoethanol 100 mmol dm-3 (i.e. addition product **6**).

4-HSA complex and **2-MPEGA**, an additional experiment was carried out using a **2-MPEGA**-HSA 1:1 mixture but, as in the absence of HSA, no reduction by the biliverdin reductase system was observed.

Thiol addition

In this assay, each of the biliverdins under study (i.e. **4** and **2-MPEGA**) as about 36 μmol dm^{-3} aqueous solution (containing 2% DMSO for **4**) was reacted with increasing amounts of 2-mercaptoethanol (in the approximate range 10 to 100 mmol dm^{-3}), and the formation of the corresponding product of nucleophilic addition at C10 (i.e. respectively **6** and **7-MPEGA**) determined from the increase in optical density at the maximum absorption wavelength in the system used (i.e. 401 nm and 387 nm respectively). With both substrates, the addition product turned out to be formed immediately both at 293 K and at 310 K. The lower temperature was chosen in order to displace further the equilibrium to the rubinoid product [35], while the higher temperature was intended to match the conditions of the enzymatic reduction; although only the results at 293 K are discussed, those at 310 K were qualitatively identical. Interestingly, noticeable differences were found between the equilibrium constants of thiol addition to **4** vs **2-MPEGA**. Figure 3 shows the difference absorption spectra of a number of aqueous mixtures of biliverdin-IXα, **4**, and 2-mercaptoethanol containing increasing excesses of the thiol, and figure 4 shows the spectra corresponding to analogous mixtures of the mesobiliverdin-XIIIα-MPEGA, **2-MPEGA**, and the same thiol. Comparison in figures 3 and 4 of the changes in the spectra produced by thiol concentrations ca. 10 and 20 mmol dm^{-3} (i.e. spectra 1 and 2 in each figure) clearly illustrates that thiol addition is slightly less favourable for **2-MPEGA**. At 293 K and under the working conditions, an approximately 250 molar excess of thiol was needed in order to obtain an equimolar mixture of biliverdin-IXα and biliverdin-thiol adduct (i.e. 50% conversion) while about 500 molar excess of thiol was needed to achieve the same conversion from **2-MPEGA**.

This result is in agreement with the observation that, in order to chemically reduce **2-MPEGA** (above), both a larger excess of hydride and a higher temperature are needed relative to the chemical reduction of biliverdin-IXα.

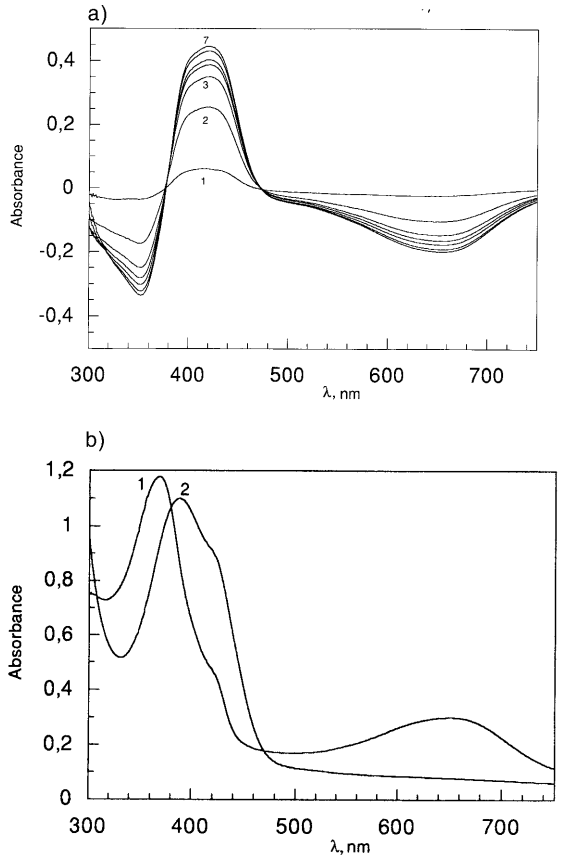

Figure 4. *a*) UV-visible spectra of the reaction of mesobiliverdin-XIIIα, **2-MPEGA**, with 2-mercaptoethanol in 0.05 mol dm^{-3} aqueous sodium phosphate buffer, pH 7.4, at 293 K. Reference cell (1 cm path): **2-MPEGA** solution 36 μmol dm^{-3}, in buffer containing 2% (v:v) DMSO. Sample cell: reference solution plus 2-mercaptoethanol. (1) 10 mmol dm^{-3}, (2) 20 mmol dm^{-3}, (3) 30 mmol dm^{-3}, (4) 40 mmol dm^{-3}, (5) 50 mmol dm^{-3}, (6) 75 mmol dm^{-3}, (7) 100 mmol dm^{-3}. *b*) UV-visible spectra vs buffer solution of (1) sample cell before the addition of thiol (i.e. **2-MPEGA**) and (2) sample cell after the addition of 2-mercaptoethanol 100 mmol dm^{-3} (i.e. addition product **7-MPEGA**).

Conclusions

Both biliverdin-IXα, **4**, and the mesobiliverdin derivative **2-MPEGA** react readily with 2-mercaptoethanol in water at 310 K to add the thiol reversibly to the C10 position of each verdin (and the equilibria are even more shifted towards the adduct at 293 K); however, while biliverdin-IXα – as its 1:1 complex to HSA – is efficiently reduced by the biliverdin reductase system under essentially the same conditions, the mesobiliverdin derivative **2-MPEGA** is inert towards the same reaction. Since the addition of the biliverdin reductase thiol group to position 10 of biliverdin is the initial step in the reaction sequence leading to bilirubin, the present results allow us to discount an intrinsically low reactivity of **2-MPEGA** towards thiols as the reason for the apparent inertness of this mesobiliverdin to the enzymatic reduction. It must instead be attributed to the inability of this particular mesobiliverdin derivative to fit properly into the enzyme binding site, either because of the steric hindrance imposed by its two polymeric chains or by its lack of (one or) two propionic acid groups in positions C8 and (or) C12 of the molecule, as in the natural biliverdin-IXα.

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