Synthesis of Biliverdin and Bilirubin 1-*O*-Acyl- β -D-glucopyranuronic Acids

Frans COMPERNOLLE

Laboratory of Hepatology and Laboratory of Organic Chemistry, University of Leuven, B-3000 Leuven, Belgium

(Received 19 November 1979)

Biliverdin and bilirubin mono- and di- β -glucuronides were prepared by nucleophilic substitution of the 1-*O*-mesyl derivative of α -ethoxyethyl-protected glucuronic acid (compound II) with the tetrabutylammonium salts of biliverdin and bilirubin. Removal of the acetal-protecting groups by mild acid treatment yielded biliverdin glucuronides, which were reduced to bilirubin glucuronides. Depending on reaction conditions the pure β -anomers or mixtures highly enriched in the β -anomers were obtained. The biliverdin and bilirubin glucuronides were identical with pigments derived from bile. They were characterized as the IX α isomers and the β -anomers by alkaline hydrolysis, n.m.r. spectroscopy, hydrolysis with β -glucuronidase and conversion into dipyrrolic azopigments. Model reactions of the 1-*O*-mesylate (II) with other nucleophiles also were performed, i.e. the acetate anion and various alcohols.

Bilirubin mono- and di- β -glucuronides are the main excretion products of bilirubin in bile of man and rat (Heirwegh *et al.*, 1975; Gordon *et al.*, 1976, 1977; Blumenthal *et al.*, 1977, 1979). Structures of the glucuronides and their 2-, 3- and 4-*O*-acyl rearrangement products in bile have been established (Compernelle *et al.*, 1970, 1978*a,b*; Blanckaert *et al.*, 1978; Compernelle, 1978).

Several attempts at their chemical synthesis have been reported by Thompson & Hofmann (1976), Hancock *et al.* (1976) and Compernelle (1980*a*). Recently, an acetalized derivative of glucuronic acid with a free C-1 hydroxy group was prepared (Compernelle, 1980*b*). Esterification of this acetal (compound I) with bilirubin di-imidazole, oxidation with DDQ and mild acid hydrolysis yielded biliverdin 1-*O*-acyl mono- and di-glucuronides, present as 1:1 anomeric mixtures (Compernelle, 1980*c*). Bilirubin glucuronides were produced by reduction of biliverdin derivatives with NaCNBH₃. The mono-glucuronide anomers of biliverdin and bilirubin were separated by t.l.c., but the more complex anomeric mixtures of diglucuronides (α,α , α,β and β,β) were not.

The present paper reports the specific synthesis of the β -glucuronides of biliverdin and bilirubin.

Results and Discussion

Esterification of the free C-1 hydroxy group in benzyl-, allyl- or α -ethoxyethyl-protected de-

Abbreviation used: DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

rivatives of glucuronic acid yields anomeric mixtures enriched in the α -form, if non-bulky reagents, e.g. acetic anhydride, are used (Keglevic *et al.*, 1968; Compernelle, 1980*a,b*). The proportion of β -anomer increases by using higher temperatures (Compernelle, 1980*a,c*) or sterically more demanding reagents such as mesitoyl chloride or carboxylic acids activated with dicyclohexylcarbodi-imide (Keglevic *et al.*, 1968). However, the use of the latter compound as a condensing reagent for bilirubin and biliverdin was unsuccessful, owing to the formation of *N*-acylureas (Hancock *et al.*, 1976; F. Compernelle, unpublished work).

Transformation of the C-1 hydroxy group of compound (I) to a good leaving group should allow displacement by carboxylate anions and other nucleophiles. Further, the C-2 acetal oxygen atom could participate in the formation of a C-1 carbonium ion, thus preventing attack of the nucleophile from the α -side. Treatment of compound (I) with mesyl chloride and *s*-collidine in dichloromethane yielded the 1-*O*-mesyl derivative (II) (Scheme 1), representing probably an anomeric mixture enriched in the α -isomer. The mass spectrum of compound (II) showed the characteristic $[M-\text{CHMeOEt}]^+$ ion at m/e 487. The 1-*O*-mesylate was made to react with tetrabutylammonium acetate in dichloromethane (Scheme 1), the acetal-protecting groups were removed by mild acid treatment and the reaction products were silylated. Analysis by g.l.c.-mass spectrometry revealed the presence of the β -anomer of 1-*O*-acetyl-D-glucopyranuronic acid and of the α -anomer

Scheme 1. *Suggested mechanism for formation of β -glucuronides*

Compound (I) ($R = -CHMeOEt$) is converted into 1-*O*-mesylate (II). Nucleophilic substitution of the 1-*O*-mesyl group with various nucleophiles, i.e. acetate anion, methanol, ethanol, propan-2-ol, *t*-butyl alcohol and biliverdin and bilirubin di-anions proceeds either via S_N2 reaction or via intermediate (III). β -Glycosides and α -ethyl glucoside are produced from intermediate (III), whereas S_N2 reaction of the α - and β -anomers of mesylate (II) gives β - and α -glycosides respectively. Abbreviation used: Nu, nucleophile.

of ethyl D-glucopyranosiduronic acid. Relative amounts were 2:1 respectively; other anomers were not detected.

With a large excess of methanol as the nucleophile, rapid reaction occurred at 22°C, producing (after hydrolysis) β -methyl-, α -ethyl- and a small amount of α -methyl-D-glucopyranosiduronic acid in the relative proportions 6:3:1. With ethanol, propan-2-ol and *t*-butyl alcohol the ratio β -alkyl glycoside/ α -ethyl glycoside was 7:3, 1:1, and 1:4 respectively; no α -isopropyl and α -*t*-butyl glycosides were detected. Decreasing nucleophilicity of alcohols was reflected also by the presence of increasing amounts of glucuronolactone and glucuronic acid, formed by hydrolysis of the unreacted 1-*O*-mesylate (II).

The results can be explained as follows (Scheme 1). Nucleophilic attack on intermediate ion (III) could occur at either one of two carbon atoms bearing a positively charged oxygen atom, i.e. C-1 and the acetal carbon atom at C-2. This eventually gives rise to β -acetyl (or β -alkyl) and α -ethyl glycosides respectively. Participation of the C-2 acetal oxygen atom probably enhances formation of ion (III) from the β -mesylate. Direct S_N2 displacement of the α -mesylate also would contribute to the

β -glycosides (Scheme 1), to an extent dependent on reactivity and concentration of the nucleophile. The small amount of α -methyl glycoside formed with methanol likewise can be explained by S_N2 reaction of the β -mesylate. Hence, β -glycosides seem to be produced with higher stereospecificity (but lower yields, owing to formation of the α -ethyl glycoside), if reaction proceeds via intermediate (III), i.e. for low concentration and/or reactivity of the nucleophile (see also below). Under the latter conditions the β -mesylate will react exclusively via intermediate (III), whereas β -glycosides are formed from the α -mesylate either via intermediate (III) or by S_N2 reaction.

The tetrabutylammonium salt of biliverdin was reacted with the mesylate (II) under two different conditions. Reaction in dichloromethane solution was carried out at 22°C for 1 day in the presence of molecular sieves (traces of water apparently inhibit the reaction). Alternatively, the solution was evaporated and the residue was heated at 60°C for 10 min. At this stage t.l.c. indicated formation of mono- and mainly di-esters of biliverdin. The esters formed represent glycosides (reaction at C-1) and acetal esters (reaction at the C-2 acetal function) (Scheme 1). Subsequent treatment with mild acid

removed the acetal-protecting groups, but also cleaved the acetal ester linkages with biliverdin, giving rise to biliverdin and biliverdin monoglucuronide as the main reaction products. Mono- and di-glucuronides of biliverdin were isolated by using preparative t.l.c. Yields were 6.1 and 1.5% respectively for the first (22°C) procedure and 15 and 5.7% respectively for the second (60°C) procedure.

Analytical t.l.c. of biliverdin monoglucuronide prepared at 22°C showed the presence of the β -anomer only; variable amounts (10–25%) of α -monoglucuronide were observed when the esterification was performed at 60°C. Treatment with β -glucuronidase gave complete hydrolysis to biliverdin for biliverdin mono- and di-glucuronides prepared at 22°C. Biliverdin mono- α -glucuronide resisted the action of β -glucuronidase. Biliverdin diglucuronide prepared at 60°C was hydrolysed to biliverdin (66%) and biliverdin α -monoglucuronide (34%). This represents about 83% of β -linkages. No hydrolysis was observed in control experiments, in which β -glucuronidase was either omitted or inhibited by glucaro-1,4-lactone.

The n.m.r. spectra of biliverdin mono- and di-glucuronides showed signals for the anomeric protons (β -form) at δ 5.35 p.p.m. with $J_{1,2}$ 7 Hz. Other signals were due to the ring protons of the sugar (δ 3.1–4.1 p.p.m.) and to the biliverdin aglycone methyl groups (δ 2.11 and 2.17 p.p.m.), propionic acid side chains (δ 2.95 p.p.m.) and methine protons (δ 6.12 p.p.m.).

Hydrolysis of the glucuronides with 0.1 M-NaOH yielded biliverdin, which was reduced to bilirubin. T.l.c. revealed a III α /IX α /XIII α -isomer composition of 2:93:5. This was similar to the composition (2.5:92:5.5) found for the sample (Merck A.-G.) of bilirubin that was used for preparation of biliverdin (McDonagh, 1979). These small percentages of isomers III α and XIII α are possibly not significant, as some dipyrrole exchange always occurs during application of bilirubin to t.l.c. plates (McDonagh & Assisi, 1971, 1972).

The biliverdin glucuronides were reduced readily to the corresponding bilirubin glucuronides with sodium cyanoborohydride in propan-2-ol/acetic acid (9:1, v/v). When stored (0°C in the dark) with a small amount of the reducing agent, these solutions were fairly stable with respect to oxidation and dipyrrole exchange. Bilirubin glucuronides were isolated with t.l.c., but dipyrrole exchange occurred during evaporation of eluates (as shown by disproportionation of the monoglucuronide), unless protective agents such as EDTA, ascorbic acid or NaCNBH₃ were added.

Esterification of the 1-*O*-mesyl compound (II) with the tetrabutylammonium salt of bilirubin was performed at 60°C under reaction conditions similar to those described for biliverdin. Acetal groups were

removed with acetic acid/aqueous 0.1 M-HCl (4:1, v/v) in the presence of ascorbic acid. Both mono- and di-glucuronide of bilirubin were isolated by t.l.c. in various proportions (in one experiment yields were 4.0 and 3.3% respectively). Disproportionation of bilirubin monoglucuronide during acid hydrolysis of acetal groups was shown to be responsible for the relatively high but varying proportion of diglucuronide. First, alkaline hydrolysis of the glucuronides revealed the presence of bilirubin isomers III α , IX α and XIII α in the ratio of approx. 1:2:1. Further, oxidation of bilirubin esters preceding removal of acetal groups gave only a trace amount of biliverdin diglucuronide. Biliverdin and biliverdin mono- β -glucuronide were detected as the main components; the β / α -anomer ratio for the monoglucuronide was about 20:1 to 9:1, depending on reaction time for esterification.

One may conclude that bilirubin β -glucuronides can be prepared as the IX α -isomers by starting from either biliverdin or bilirubin. In each case acetal groups are removed from biliverdin ester intermediates and the final step consists of reduction of biliverdin glucuronides.

The bilirubin glucuronides were characterized further as the ethyl anthranilate azopigments and their methyl esters. The diazonium reagent cleaved the bilirubin tetrapyrroles to dipyrrolic azopigments, yielding an equimolar mixture of azodipyrrole acid and azodipyrrole glucuronide from bilirubin monoglucuronide; only the glucuronide azopigment was obtained from bilirubin diglucuronide. Brief treatment with diazomethane converted azodipyrrole glucuronides into the methyl esters. (Longer reaction times gave rise to base-catalysed acyl shift of the azodipyrrole α -1-*O*-acyl group, producing the methyl ester of 2-*O*-acyl azodipyrrole glucuronide from the more reactive α -anomer.) T.l.c. of the methyl esters confirmed that β -glucuronides were formed either exclusively or predominantly (22 and 60°C reaction conditions respectively).

The electron-impact mass spectra of the trimethylsilyl ether methyl ester derivatives of azodipyrrole β -glucuronide showed the expected molecular ions at m/e 796 and 868, corresponding to introduction of two and three silyl groups (Compernelle *et al.*, 1978a,b).

T.l.c. behaviour of biliverdin, bilirubin and azodipyrrole glucuronide obtained by synthesis (including the methyl ester of the azopigment) was identical with that observed for pigments prepared from natural sources. Reference biliverdin β -glucuronides were derived from natural β -glucuronides by oxidation with DDQ.

Conclusion

Methods were developed recently for the syn-

thesis of the acetal-protected derivative (I) of glucuronic acid (Compernelle, 1980b) and for esterification of compound (I) with bilirubin diimidazole, yielding anomeric mixtures of biliverdin and bilirubin glucuronides (Compernelle, 1980c). The present paper describes conversion of compound (I) into the 1-*O*-mesylate (II) and substitution of the C-1 mesylate group by various nucleophiles, i.e. acetate anion, alcohols and biliverdin and bilirubin di-anions. Depending on reaction conditions, biliverdin and bilirubin glucuronides are isolated as the pure β -anomers or as anomeric mixtures highly enriched in the β -anomers. Participation of the C-2 acetal group appears to play a key role in the formation of β -glycosidic and C-2 acetal esters, although glycosides may also be formed by S_N2 reaction.

Chemical manipulations, starting from biliverdin and bilirubin, are simple and straightforward. Yet large-scale purification of the polar and sensitive tetrapyrrole conjugates remains a difficult task, perhaps to be effected by high-pressure liquid chromatography.

Experimental

Materials

Chemicals were purchased as described previously (Compernelle *et al.*, 1978a). Bilirubin, DDQ and tetrabutylammonium hydroxide (0.1 M solution in methanol/propan-2-ol) were from E. Merck A.-G. (Darmstadt, Germany). Methanesulphonyl chloride (mesyl chloride) and 2,4,6-trimethylpyridine (*s*-collidine, dried over KOH pellets and distilled before use; b.p. 169–172°C) were obtained from Fluka A.-G. (Buchs, Switzerland). Biliverdin was prepared as reported by McDonagh (1979). α -Ethoxyethyl 2,3,4-tri-*O*-(α -ethoxyethyl)-D-glucopyranuronate (compound I, a mixture of diastereoisomers) was prepared as described by Compernelle (1980b). β -Glucuronidase and glucaro-1,4-lactone were obtained as reported by Blanckaert *et al.* (1977).

Apparatus and chromatography systems

N.m.r. spectra. Spectra for biliverdin glucuronides were recorded at 100 MHz with a Varian XL-100 spectrometer in $[^2\text{H}]$ dimethyl sulphoxide/ $[^2\text{H}]$ acetic acid (5:1, v/v) by accumulation in the continuous wave mode.

Mass spectra. Spectra of the trimethylsilyl ether methyl ester of azodipyrrole β -D-glucuronide were run as reported for azopigments prepared from bile (Compernelle *et al.*, 1978a,b). G.l.c.-mass spectrometry was carried out as described previously (Compernelle, 1980a,b).

Thin-layer chromatography. The following solvent systems were employed: A, chloroform/methanol/water/acetic acid (100:50:10:1, by

vol.); B, chloroform/methanol (9:1, v/v); C, chloroform/acetic acid (99:1, v/v). Precoated silica gel plates (F254 from Merck A.-G.; glass or aluminium sheets; layer thickness 0.25 and 0.20 mm) were utilized for analytical t.l.c. For preparative t.l.c. the same plates or self-prepared plates (silica gel PF 254 from Merck A.-G.; layer thickness 1 mm) were used.

Methods

α -Ethoxyethyl 2,3,4-tri-*O*-(α -ethoxyethyl)-1-*O*-mesyl-D-glucopyranuronate (compound II). Compound I (30 mg) was dissolved in dichloromethane (5 ml) and *s*-collidine (0.3 ml). After mixing the cooled solution (0°C) with methanesulphonyl chloride (50 μ l) and reaction at 0°C for 10 min, the organic layer was shaken vigorously with a saturated $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer solution (pH 9.0; 6 $\times$ 5 ml; 22°C). The dichloromethane layer was washed with water (3 $\times$ 5 ml) and dried over molecular sieves (0.4 nm pore diameter) for 2 h. The mass spectrum of the labile mesylate (II) showed peaks at *m/e* 487 corresponding to the ion $[\text{M}-\text{CHMeOEt}]^+$ and further at *m/e* 437, 415, 397, 391, 365, 347, 320 and 319.

Reaction of mesylate (II) with tetrabutylammonium acetate and alcohols. Tetrabutylammonium acetate was prepared from acetic acid (6 mg) and 0.1 M-tetrabutylammonium hydroxide (1 ml). The solution was evaporated to dryness *in vacuo*, the residue was dissolved in dichloromethane (1 ml) and mesylate (II) (prepared from 5 mg of compound I) in dichloromethane (1 ml) was added. After 1 day at 22°C the organic layer was washed with water (5 $\times$ 3 ml), evaporated, and hydrolysed with acetic acid/aqueous 0.1 M-HCl (4:1, v/v) for 30 min. The solution was evaporated at 22°C *in vacuo* and the residue was silylated by addition of *NO*-bis(trimethylsilyl)trifluoroacetamide (50 μ l) and pyridine (100 μ l) in this order. G.l.c.-mass spectrometry revealed the presence of β -acetyl and α -ethyl glucopyranosiduronic acids (ratio, 2:1) as the main constituents.

Reaction of mesylate (II) with methanol, ethanol, propan-2-ol and *t*-butyl alcohol was carried out by addition of excess alcohol (2 ml) to a dichloromethane solution (1 ml) of the mesylate (prepared from 5 mg of compound I). After reaction for 1 day (methanol) or 2 days (other alcohols) at 22°C the solutions were evaporated *in vacuo*. Hydrolysis and silylation were performed as described above.

Elution temperatures of silylated alkyl and acetyl D-glucopyranosiduronic acids were as follows (3% OV101, programme, 150°C, 4°C/min): α -methyl, 165°C; β -methyl, 167°C; α -ethyl, 167°C; β -ethyl, 169°C; β -isopropyl, 170.5°C; β -*t*-butyl, 172.5°C; β -acetyl, 172°C. The α -acetyl glucuronide was prepared in an independent way (Compernelle, 1980b), and was eluted at 170°C. With the sterically

hindered alcohols incomplete reaction was revealed by detection of $\alpha\beta$ -lactone (159.5°C) and α - and β -glucopyranuronic acids (168 and 172.5°C). The mass spectra of alkyl and acetyl glucuronides confirmed the structures assigned, i.e. the nature of the C-1 substituent and the pyranose ring form. Characteristic ions $[M-CH_3]^+$ and $[M-CH_3-Me_3SiOH]^+$ and further abundant peaks at m/e 305, 217 and 204 were detected.

Biliverdin glucuronides. Biliverdin (6.0 mg) was converted into the di-anion by addition of 0.1 M-tetrabutylammonium hydroxide (0.21 ml) and dichloromethane (1 ml). The solution was evaporated to dryness *in vacuo* (60°C, rotation pump). The residue was dissolved in dichloromethane (5 ml) containing mesylate (II) (prepared from 30 mg of compound I). Molecular sieve (pore size 0.4 nm) was added and after 1 day at 22°C the reaction mixture was evaporated *in vacuo* (30°C, rotation pump).

Alternatively, addition of molecular sieve was omitted, and after evaporation of solvent and *s*-collidine the residue was heated *in vacuo* (60°C, rotation pump) for 10 min. For both reaction conditions t.l.c. (solvent system B) revealed formation of mainly di-esters (R_F 0.72 and 0.69), and smaller amounts of mono-esters (R_F 0.22) and biliverdin (R_F 0.02). The residue was hydrolysed with acetic acid (1 ml) and aqueous 0.1 M-HCl (0.25 ml) at 22°C for 30 min, followed by evaporation *in vacuo* (22°C, rotation pump). For preparative t.l.c. (solvent system A) the biliverdin compounds were dissolved in a minimal amount of acetic acid and 9 vol. of propan-2-ol. Biliverdin (R_F 0.82), biliverdin mono- β -glucuronide (R_F 0.33) and biliverdin diglucuronide (R_F 0.13) were the main components. Biliverdin mono- α -glucuronide (R_F 0.36) was detected as a minor component only after 60°C reaction conditions. The pigments were eluted with methanol/acetic acid (3:1, v/v). Yields (based on biliverdin) were determined by measuring absorbances at λ_{max} , 376 and 666 nm, assuming $\epsilon = 50\,800$ and $14\,400$ litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ respectively as for biliverdin (McDonagh, 1979). The eluates were evaporated *in vacuo* at 22°C.

Hydrolysis of biliverdin glucuronides with β -glucuronidase. The pigment (about 10–100 nmol) was first dissolved in 10–20 μ l of dimethyl sulphoxide and further diluted with 2 ml of acetate buffer, pH 5.2; after centrifugation one-third of the pigment solution was incubated at 37°C for 90 min with 130 μ g of β -glucuronidase in 20 μ l of water. Parallel controls were prepared by (a) omission of enzyme and (b) addition of 20 mg of glucaro-1,4-lactone dissolved in buffer. After acidification with 5 ml of glycine/HCl buffer (pH 1.8, saturated with NaCl) and extraction into 0.5 ml of butanol, the degree of conversion of biliverdin glucuronides into biliverdin

(and into biliverdin mono- α -glucuronide for biliverdin diglucuronide prepared under 60°C reaction conditions) was assessed by t.l.c. (solvent system A; isolation and absorbance measurement as described above). Under the t.l.c. conditions used biliverdin was converted slowly into a more mobile violet pigment and therefore was quantified in separate runs at an early stage of the chromatography.

Alkaline hydrolysis of biliverdin glucuronides and conversion into bilirubin. Biliverdin glucuronides were dissolved in 0.1 M-NaOH (argon atmosphere). After 1 h at 22°C the solutions were acidified with glycine/HCl buffer, pH 1.8, and biliverdin was extracted with butanol. The butanol solution was evaporated *in vacuo* and biliverdin was dissolved in acetic acid and reduced with a small amount of NaCNBH $_3$. After dilution with chloroform and washing with water, bilirubin-III α , -IX α and -XIII α isomers were determined by using t.l.c. (solvent system C) and measuring absorbances of eluates at λ_{max} , 450 nm.

Reduction of biliverdin glucuronides to bilirubin glucuronides. Biliverdin glucuronides were dissolved in a minimal amount of acetic acid. After dilution with 9 vol. of propan-2-ol (or methanol) the solution was shaken with a small amount of NaCNBH $_3$ for a few minutes until complete reduction to bilirubin glucuronides occurred. At this stage no dipyrrole exchange was observed on t.l.c. (solvent system A) for bilirubin mono- α -glucuronide (R_F 0.50) and mono- β -glucuronide (R_F 0.46). Bilirubin diglucuronide (pure β -anomers or mixtures enriched in the β -anomers) showed an R_F value of 0.12. The pigments were isolated by t.l.c. with solvent system A (acetic acid may be omitted from this solvent system, if no α -glucuronides are present), and eluted with methanol or methanol/acetic acid (9:1, v/v). Yields were determined by measuring A_{450} values of the eluates, assuming $\epsilon = 61\,500$ litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ as for bilirubin. Although reduction with NaCNBH $_3$ gave a quantitative yield of bilirubin glucuronides, losses associated with the t.l.c. isolation procedure decreased yields to 50–70%. To minimize dipyrrole exchange during evaporation of eluates, small amounts of EDTA (disodium salt) and ascorbic acid or NaCNBH $_3$ were added.

Conversion of bilirubin glucuronides into azodipyrrole glucuronide and methyl ester. After reduction of biliverdin glucuronides with NaCNBH $_3$ and dilution of the propan-2-ol/acetic acid (9:1, v/v) solutions with ethanol (4 ml) and glycine/HCl buffer, pH 2.7 (2 ml), excess of ethyl anthranilate diazonium reagent was added (Van Roy & Heirwegh, 1968). Azodipyrrole glucuronide was isolated by t.l.c. with solvent system A. The azopigment was eluted from the silica gel by shaking with pentan-2-one and glycine/HCl buffer, pH 2.7. The pentan-2-one layer was washed with water and treated briefly (1–3 s)

with ethereal diazomethane, immediately followed by evaporation *in vacuo*. T.l.c. with solvent system B (two developments) showed that the slower moving methyl ester (identical with the azodipyrrole β -glucuronide methyl ester derived from bile) was present exclusively or predominantly (22 and 60°C reaction conditions). The α -anomer exhibited a slightly higher R_F value. Any acyl shift to the 2-position of glucuronic acid that may occur for the α -anomers of biliverdin, bilirubin and azodipyrrole glucuronide (for instance, by omission of acetic acid from solvent system A, or by longer exposure to diazomethane) was detected by appearance of the faster moving 2-*O*-acyl azodipyrrole glucuronide described previously (Compennolle *et al.*, 1978a; Blanckaert *et al.*, 1978).

Reaction of mesylate (II) with bilirubin di-anion. Bilirubin (6mg) was converted into the bistetra-butylammonium salt as described for biliverdin. Reaction with mesylate (II) (prepared from 30mg of compound I) was performed at 60°C (dark, vacuum) for 10, 30 and 60 min. The reaction mixture was dissolved in dichloromethane (6ml). T.l.c. with solvent system B revealed progressive conversion of bilirubin into more polar bilirubin esters (about 50, 25 and 10% of bilirubin was left unreacted after 10, 30 and 60min). The bilirubin esters were oxidized to biliverdin derivatives by shaking the dichloromethane solutions with a freshly prepared solution of DDQ (6mg) in water for about 2min, followed by thorough washing with water (6 $\times$ 5ml). T.l.c. with solvent system B confirmed progressive formation of biliverdin diesters and monoesters, which exhibited R_F values identical with those mentioned above (see under 'Biliverdin glucuronides'). Hydrolysis of acetal groups was carried out as described above, yielding biliverdin and biliverdin mono- β -glucuronide as the main reaction products. Trace amounts of biliverdin mono- α -glucuronide and diglucuronide also were detected.

Alternatively, bilirubin esters were hydrolysed directly with acetic acid and aqueous 0.1M-HCl containing ascorbic acid (4:1, v/v) for 30min (dark, argon, 22°C). Bilirubin pigments were extracted by addition of propan-2-ol (1 vol.), chloroform (2 vol.) and glycine/HCl buffer (pH 1.8, saturated with NaCl). Preparative t.l.c. with solvent system A yielded bilirubin mono- and di-glucuronides, present in varying relative amounts.

Alkaline hydrolysis of bilirubin glucuronides. Silica gel powders containing the bilirubin glucuronides were shaken with 3ml of 1M-NaOH [containing 50mg of ascorbic acid, a trace amount of EDTA (disodium salt), and flushed with argon]. The

suspension was kept in the dark under argon for 30min. With a Pasteur pipette the yellow aqueous solution was poured into a mixture of chloroform (1ml) and acetic acid (0.3ml). After washing with water, the bilirubin solution was analysed with t.l.c. (solvent system C).

I thank Professor K. P. M. Heirwegh, Professor G. Hoornaert, Dr. D. W. Hutchinson and Dr. N. Blanckaert for their interest and encouragement, Dr. S. Toppet for running the n.m.r. spectra, Mr. P. Leroy, Mr. R. Es and Mr. R. De Boer for technical assistance, and the National Foundation for Scientific Research of Belgium for financial support.

References

- Blanckaert, N., Fevery, J., Heirwegh, K. P. M. & Compennolle, F. (1980c) *FEBS Lett.* in the press
- Blanckaert, N., Compennolle, F., Leroy, P., Van Houtte, R., Fevery, J. & Heirwegh, K. P. M. (1978) *Biochem. J.* **171**, 203–214
- Blumenthal, S. G., Taggart, D. B., Ikeda, R. M., Ruebner, B. H. & Bergstrom, D. E. (1977) *Biochem. J.* **167**, 535–548
- Blumenthal, S. G., Taggart, D. B., Rasmussen, R. D., Ikeda, R. M. & Ruebner, B. H. (1979) *Biochem. J.* **179**, 537–547
- Compennolle, F. (1978) *Biochem. J.* **175**, 1095–1101
- Compennolle, F. (1980a) *Carbohydr. Res.* in the press
- Compennolle, F. (1980b) *Carbohydr. Res.* in the press
- Compennolle, F. (1980c) *FEBS Lett.* in the press
- Compennolle, F., Jansen, F. H. & Heirwegh, K. P. M. (1970) *Biochem. J.* **120**, 891–894
- Compennolle, F., Van Hees, G. P., Blanckaert, N. & Heirwegh, K. P. M. (1978a) *Biochem. J.* **171**, 185–201
- Compennolle, F., Van Hees, G. P. & Heirwegh, K. P. M. (1978b) *Biomed. Mass Spectrom.* **5**, 453–459
- Gordon, E. R., Goresky, C. A., Chan, T.-H. & Perlin, A. S. (1976) *Biochem. J.* **155**, 477–486
- Gordon, E. R., Chan, T.-H., Samodai, K. & Goresky, C. A. (1977) *Biochem. J.* **167**, 1–8
- Hancock, F. E., Hutchinson, D. W. & Knell, A. J. (1976) *Biochem. Soc. Trans.* **4**, 303–304
- Heirwegh, K. P. M., Fevery, J., Michiels, R., Van Hees, G. P. & Compennolle, F. (1975) *Biochem. J.* **145**, 185–199
- Keglevic, D., Pravdic, N. & Tomasik, J. (1968) *J. Chem. Soc. C* 511–514
- McDonagh, A. F. (1979) in *The Porphyrins* (Dolphin, D., ed.), Biochemistry, Part A, pp. 293–491, Academic Press, New York
- McDonagh, A. F. & Assisi, F. (1971) *FEBS Lett.* **18**, 315–317
- McDonagh, A. F. & Assisi, F. (1972) *Chem. Commun.* 117–119
- Thompson, R. P. H. & Hofmann, A. H. (1976) *Biochim. Biophys. Acta* **451**, 267–277
- Van Roy, F. P. & Heirwegh, K. P. M. (1968) *Biochem. J.* **107**, 507–518