Structure Revision of Disaccharidic Conjugates of Bilirubin-IX α in Human Bile and Identification of Phenylazo Derivatives B₄, B₅ and B₆ as 2-, 3- and 4-*O*-Acylglucuronides

By FRANS COMPERNOLLE

Laboratory of Macromolecular and Organic Chemistry, University of Leuven, Heverlee, Belgium

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Aniline azopigments B₄, B₅ and B₆, derived from conjugates of bilirubin-IX α in human bile, and previously characterized as disaccharidic esters [Kuenzle (1970) *Biochem. J.* **119**, 387–394 and 411–435], were analysed by using t.l.c. and mass spectrometry. The compounds were identified as partially separated mixtures of 2-, 3- and 4-*O*-acylglucuronide positional isomers. The 1-*O*-acylglucuronide was not detected in the mixtures and was the only compound hydrolysed with β -glucuronidase. Further scrutiny of structural assignments made by Kuenzle [(1970) *Biochem. J.* **119**, 411–435] led to identification of the lactone and hexuronic acid derivatives that were obtained from azopigment B₅ along with glucuronolactone and glucuronic acid. A branched-chain structure, i.e. 3-*C*-hydroxy-methyl-D-riburonic acid, was assigned previously, but the derivatives have now been identified as various incompletely silylated forms of glucuronolactone and glucuronic acid. Several trimethylsilyl derivatives of glucuronolactone were isolated and characterized by n.m.r. and mass spectrometry.

Contradictory results have been reported for the structures of conjugates of bilirubin-IX α . Kuenzle (1970*a,b*) assigned disaccharidic structures, i.e. aldobiouronic acid, hexuronosylhexuronic acid and pseudoaldobiouronic acid, to the aniline azopigments B₄, B₅ and B₆ derived from human bile, and failed to detect a simple glucuronide.

Compernelle *et al.* (1970) assigned a glucuronide structure to a hexuronic acid-containing azopigment (called azopigment δ) derived from rat bile. The glucuronide structure of the same azopigment derived from human, rat and dog bile has been confirmed (Compernelle *et al.*, 1975, 1977, 1978; Gordon *et al.*, 1976, 1977). However, whereas the 1-*O*-acylglucopyranuronoside structure applies to bilirubin conjugates present in fresh normal bile, obstruction of bile flow or incubation of bile under N₂ results in a base-catalysed sequential rearrangement of the bilirubin-IX α acyl group to positions 2, 3 and 4 of glucuronic acid (Compernelle *et al.*, 1978; Blanckaert *et al.*, 1978). This results in a complex composition of the ethyl anthranilate or aniline δ -azopigment fraction, which consists of the four positional isomers. These 1-, 2-, 3- and 4-*O*-acylglucuronides, called (Compernelle *et al.*, 1978) azopigments (I), (II), (III) and (IV), have been partially separated on silane-treated t.l.c. plates. Complete separation and structural characterization was achieved for the four methyl esters formed by reaction of the glucuronic acid carboxy group with diazomethane (Compernelle *et al.*, 1978).

The conditions utilized for isolation of the four azopigment isomers (Compernelle *et al.*, 1978) closely resembled those reported by Kuenzle (1970*a*) for isolation of azopigments B₄, B₅ and B₆. In both cases bile was treated with an aniline diazonium reagent of pH 6.0, devoid of excess of aromatic amine (using an ethyl anthranilate diazonium reagent of pH 6.0 gave very similar results). In our work rearranged glucuronide azopigments were prepared from post-obstructive or incubated bile. In Kuenzle's work (1970*a*) massive amounts of bile were collected from patients operated for gallstones and these were stored in a deep-freeze for unspecified periods of time. The conditions mentioned, and also the initial stages of the reversed-phase chromatography of bile (pH about 7.8) at 20°C with a dilute buffer of pH 6.0, should give rise to migration of the bilirubin acyl group. Blanckaert *et al.* (1978), who studied the kinetics of the base-catalysed, sequential (1 $\rightarrow$ 2, 2 $\rightarrow$ 3, 3 $\rightarrow$ 4) rearrangement, demonstrated that the reaction proceeds by treatment of either bile or isolated bilirubin glucuronides with buffers of pH 6–9.

The suggestion that azopigments B₄, B₅ and B₆ are rearranged glucuronides (Compernelle *et al.*, 1978) has now been verified by analysis of authentic samples. T.l.c. mobilities and mass spectra were identical for our samples and samples provided by Dr. C. C. Kuenzle (University of Zürich), proving that either the glucuronide or the disaccharide structural proposal is wrong. The 1-*O*-acylglucuronide and rearranged 2-, 3- and 4-*O*-acylglucu-

ronide structures have been discussed thoroughly (Compernelle *et al.*, 1978). In the present work structural assignments by Kuenzle (1970b) are scrutinized.

One important aspect is concerned with identification of an unknown hexuronic acid obtained from azopigment B₅. A branched-chain structure, i.e. 3-C-hydroxymethyl-D-riburonic acid, has been proposed on the basis of the mass spectra of various trimethylsilyl derivatives of the unknown hexuronic acid (Kuenzle, 1970b). However, g.l.c. retention times and mass spectra of trimethylsilyl derivatives of 3-C-hydroxymethyl-D-riburonic acid, obtained by synthesis (Paulsen & Stenzel, 1974; Blackstock *et al.*, 1974), differed from those obtained for trimethylsilyl derivatives of the unknown hexuronic acid. The unknown compounds are now identified as incompletely silylated derivatives of glucuronolactone and glucuronic acid.

Materials and Methods

Chemicals

Samples of aniline azopigments B₄, B₅ and B₆ were kindly provided by Dr. C. C. Kuenzle (University of Zürich). β -Glucuronidase, glucuronic acid, glucuronolactone, glucaro-1,4-lactone and *NO*-bis(trimethylsilyl)trifluoroacetamide were purchased as specified in previous papers (Blanckaert *et al.*, 1977; Compernelle *et al.*, 1978).

Analysis of azopigments B₄, B₅ and B₆

For t.l.c. of the unmodified azopigments B₄, B₅ and B₆, pre-coated F254 silica-gel plates (layer thickness 0.25 mm; Merck A.-G., Darmstadt, Germany) were treated with dichlorodimethylsilane (Compernelle *et al.*, 1978). The plates were developed with chloroform/methanol (9:1 or 17:3, v/v).

Methyl esters were prepared by dissolving the azopigments B₄, B₅ and B₆ in pentan-2-one/formamide (4:1, v/v), washing out the formamide with water, and treatment of the water-saturated organic layer with ethereal diazomethane. The solutions were evaporated *in vacuo*. T.l.c. of the methyl esters was carried out on unmodified F254 silica-gel plates with the solvent system chloroform/methanol (17:3, v/v).

Trimethylsilylation of the methyl esters of azopigments B₄, B₅ and B₆ was performed by treatment with pyridine/*NN*-bis(trimethylsilyl)trifluoroacetamide (1:1, v/v; 50 μ l) for 1 h. Mass spectra were run on an AEI MS902S mass spectrometer at ion-source temperatures of 220–250°C.

Reference azopigments, i.e. phenylazo compounds (I), (II) plus (III), and (IV), and the single four methyl ester derivatives, were prepared as described previously (Compernelle *et al.*, 1978). The azopig-

ment methyl esters moved in decreasing order of mobility: (II), (III), (I) and (IV). The same order of movement was observed for the azopigment acids on silane-treated plates, except that azopigments (II) and (III) were not separated. The azopigments B₄, B₅ and B₆ and the phenylazo compound (I) were incubated with β -glucuronidase by using the method of Blanckaert *et al.* (1977). The degree of hydrolysis was assessed by t.l.c. with the solvent system chloroform/methanol/water (65:25:3, by vol.). Untreated t.l.c. plates were used. Under these conditions azopigment acids (I), (II), (III) and (IV) are not separated.

Preparation and analysis of incompletely silylated derivatives of glucuronolactone and glucuronic acid

Silylation. For incomplete silylation solutions (a), (b), (c) and (d) were supplemented with 40 μ l of *NO*-bis(trimethylsilyl)trifluoroacetamide. Complete silylation was obtained with 200 μ l of the reagent. After 10 min the reagents were evaporated *in vacuo* (bath temperature 50°C) and the residues were dissolved in dichloromethane (2 ml). The solutions were prepared as follows: (a) glucuronic acid (10 mg) was dissolved in 0.1 M-HCl, after 10 min the solution was evaporated *in vacuo* (bath temperature 60°C), and the residue was dissolved in pyridine (0.3 ml). (b) Glucuronic acid (10 mg) and (c) glucuronolactone (10 mg) were dissolved in pyridine (0.3 ml). (d) A solution of glucuronolactone (10 mg) in pyridine (0.3 ml) and triethylamine (0.15 ml) was boiled for 1 min, cooled rapidly and, without delay, treated with the silylating reagent.

Preparative separation. Monohydroxy lactones were isolated from the incompletely silylated solution (c) (30 mg of glucuronolactone) by using high-pressure liquid chromatography on silica gel under the following conditions: Li Chrosorb SI60 (Merck A.G.), particle size 10 μ m; column length 120 cm; external diameter 9.5 mm; solvent hold-up 47.2 ml; injection volume 2 ml; flow 4 ml/min; refractive index detector. Dichloromethane was used as the solvent for isolating the α -2-hydroxy lactone (X) (elution volume 90 ml) and the $\alpha\beta$ -1-hydroxy lactones (VII) plus (VIII) (elution volume 112 ml). The latter fraction represented a mixture of the three components owing to tailing of the former compound. The more polar β -2-hydroxy lactone (IX) was purified in a separate run with dichloromethane/acetonitrile (9:1, v/v) as the solvent (elution volume 70 ml).

The β -5-hydroxy compound (XI) was isolated from the incompletely silylated solution (d) by preparative g.l.c. on 5% Apiezon (column length 1.5 m; internal diameter 9.5 mm; N₂ flow approx. 200 ml/min; programme 100–150°C, 4°C/min; flame ionization detector; outlet split ratio 1:100). Compound (XI) was eluted with *R_s* 0.85 [retention time relative

to that of β -tris(trimethylsilyl)glucuronolactone (V)]. The collection tubes were immersed in a solid- CO_2 /acetone bath and after collection the fractions were removed with dichloromethane.

G.l.c. and g.l.c.-mass spectrometry. These were performed on reaction mixtures and on purified compounds by using a 3% OV 101 column heated at 155°C and a 1% QF1 column heated at 175°C (glass columns; length 1.8 m; internal diameter 2 mm; He flow 40 ml/min). A double-stage jet separator, constructed in stainless steel, and the ion source of the AEI MS902S mass spectrometer were operated at 200°C. Alternatively, a silicone membrane separator and an AEI MS12 mass spectrometer were utilized under similar conditions. Exchange of hydroxy groups in monohydroxy lactones was carried out by introduction of $^2\text{H}_2\text{O}$ into the ion source via the heated inlet system. Results are assembled in Table 1.

N.m.r. spectrometry. N.m.r. analysis (Table 2) was carried out in [^2H]chloroform solution at 100 MHz by using a Varian XL 100 spectrometer. The signal of the [^1H]chloroform impurity was used for calibration. The spectra were run at 22°C. For the β -2-hydroxy lactone (IX) the measurements were repeated at -20°C. Spin decoupling was performed for the α - and β -2-hydroxy lactones (X) and (IX) to assign the H_2 and 2-OH protons (irradiation of H_1 , H_2 and 2-OH protons) and for the $\alpha\beta$ -1-hydroxy lactones (VII) plus (VIII) to assign the H_1 and OH protons of the α - and β -forms (irradiation of OH protons). The spectrum of the β -5-hydroxy lactone (XI) was obtained by accumulation of 100 scans. Spectra for the tris(trimethylsilyl)lactones (V) plus (VI) were run on products obtained from completely silylated solutions (c) and (d). The spectra revealed anomer ratios 7:3 and 9:1 respectively, with the β -form as the major component.

Results

Analysis of azopigments B_4 , B_5 and B_6

Thin-layer chromatography. T.l.c. clearly revealed the identity of azopigments B_4 , B_5 and B_6 with phenylazo compounds (II), (III) and (IV). Especially clear-cut results were obtained for the methyl esters. Azopigment B_4 methyl ester had the same mobility as the methyl ester of 2-*O*-acyl compound (II). For the methyl esters of azopigments B_5 and B_6 three components were observed, in each case moving (from top to bottom) as the methyl esters of 2-, 3- and 4-*O*-acyl compounds (II), (III) and (IV) respectively. The composition found for azopigment B_5 was about 60, 45 and 5% and for azopigment B_6 it was about 40, 55 and 10%. Apparently, except for azopigment B_4 , only partial separation had occurred on the reversed-phase column, resulting in progres-

sive enrichment of azopigment fractions B_5 and B_6 in the more polar components.

A spot corresponding to the 1-*O*-acylglucuronide, phenylazo compound (I), was not observed in the chromatograms of either azopigment acids or methyl esters. Otherwise t.l.c. of the azopigment acids B_4 , B_5 and B_6 confirmed the results obtained for the methyl esters, except that the 2- and 3-*O*-acyl isomers were not separated.

Assay with β -glucuronidase. Phenylazo compound (I) (1-*O*-acylglucuronide) was hydrolysed almost completely (96%) by treatment with β -glucuronidase and the hydrolysis was inhibited completely by addition of glucaro-1,4-lactone. The hydrolysis product was identified by t.l.c. as the unconjugated phenylazo-dipyrrole. The remaining 4% of material was accounted for by the presence of 4% of the 2-*O*-acyl compound (II).

In parallel assays azopigments B_4 , B_5 and B_6 were not cleaved with β -glucuronidase, and the resulting azopigments moved on t.l.c. as the starting compounds. With the t.l.c. system used, phenylazo compounds (I), (II), (III) and (IV) and azopigments B_4 , B_5 and B_6 all exhibited the same R_F value.

Mass spectrometry of methyl ester trimethylsilyl ether derivatives of azopigments B_4 , B_5 and B_6 . A satisfactory mass spectrum was not obtained for the methyl ester trimethylsilyl ether derivative of azopigment B_4 , probably owing to lack of material.

Treatment of the methyl ester of azopigment B_5 with *NO*-bis(trimethylsilyl)trifluoroacetamide and pyridine gave rise to introduction of four trimethylsilyl groups [1 h treatment: 30% of tris- and 70% of tetrakis(trimethylsilyl) derivative, M^{++} 796 and 868]. The mass spectrum was virtually identical with the spectra obtained for the methyl ester trimethylsilyl ether derivatives of phenylazo compounds (II) and (III) (Compennolle *et al.*, 1978). Trimethylsilylation of the pyrrolinone lactam group was evident from the benzylic-type ion at m/e 403, originating from β -cleavage of the propionic side chain. However, controlled evaporation in the ion source also revealed important contamination of the azopigment with a more volatile derivative of glycodeoxycholic acid or an isomeric bile acid. This was shown by molecular ions M^{++} 535, 607 and 679, corresponding to methyl ester formation of the glycine residue and introduction of one, two and three trimethylsilyl groups. Prominent fragment ions were due to loss of methyl groups (m/e 592 and 664), loss of water or trimethylsilanol (m/e 517 and 427) and further to side-chain cleavage of the bile acid combined with losses of trimethylsilanol (m/e 345, 297 and 255) (Elliott, 1972).

Contamination of azopigment B_6 with the same or an isomeric bile acid was much more severe. However, the region of the molecular ion was clean, clearly showing the molecular ion M^{++} 868, corre-

sponding to a methyl ester tetrakis(trimethylsilyl) derivative of the azopigment.

Identification of 'branched-chain' hexuronolactones X5.2, X5.3 and X5.4 as incompletely silylated glucuronolactones

When the carbohydrate fraction of azopigment B₅ was hydrolysed with 0.1M-HCl, only glucuronic acid and glucuronolactone were detected by g.l.c.-mass spectrometry of the neutralized and trimethylsilylated reaction mixture (Kuenzle, 1970b). However, when neutralization was omitted, five additional compounds (X5.1-X5.5) were detected by g.l.c. The mass spectra of the first three peaks appeared to be consistent with hexuronolactone structures. As the lactones could not be identified with any known hexuronic acid lactone, this result was considered as evidence for a new, alkali-sensitive hexuronic acid. The structure of a branched-chain hexuronic acid, i.e. 3-C-hydroxymethyl-D-riburonic acid, was proposed on the basis that only this compound could account for the formation of three or four lactones (Kuenzle, 1970b). However, when the 3-C-hydroxymethyl-D-riburonic acid in question became available by synthesis, it was shown to have nothing in common with the unknown hexuronic acid (Paulsen & Stenzel, 1974; Blackstock *et al.*, 1974). The mass spectra reported for compounds X5.1, X5.2 and X5.3 (Kuenzle, 1970b) suggest the presence of incompletely silylated hexuronolactones. Thus fragment ions, corresponding to losses of CH₃ (*m/e* 305) and of CH₃ and H₂O (*m/e* 287) provide support for a molecular ion *M*⁺⁺ 320 (not mentioned by Kuenzle, 1970b). Even more convincing is the shift with 72 atomic mass units, observed for a series of abundant fragment ions in the spectra of the unknown lactones compared with the spectra of the α - and β -tris(trimethylsilyl)glucuronolactones. This shift indicates the replacement of one trimethylsilyloxy group with

a hydroxy group (e.g. 377 $\rightarrow$ 305; 287 $\rightarrow$ 215; 259 $\rightarrow$ 187; 245 $\rightarrow$ 173; 243 $\rightarrow$ 171; 230 $\rightarrow$ 158; 217 $\rightarrow$ 145).

To test this hypothesis, incompletely silylated derivatives of glucuronolactone and glucuronic acid were prepared under various conditions (see the Materials and Methods section). Several monohydroxy lactones (Scheme 1) were isolated by using preparative g.l.c. and high-pressure liquid chromatography and their structures were determined with n.m.r. spectrometry (Table 2). Analysis with g.l.c.-mass spectrometry was carried out under conditions similar to those reported (Kuenzle, 1970b). Comparison of retention times (Table 1) and mass spectra (see below) revealed the identity of compounds X5.2, X5.3 and X5.4 with the monohydroxy lactones shown in Table 1. The mass spectra determined for the very weak g.l.c. peaks, eluted at the approximate position of the trace component X5.1, were also identical with the spectrum reported (Kuenzle, 1970b). However, these spectra, characterized by intense peaks at *m/e* 258 and 243, appear to be due to a continuous background, observed especially after evaporation and incomplete silylation of 0.1M-HCl solutions of glucuronic acid. Probably these compounds are formed at the injection port of the g.l.c. system by thermal loss of CO₂ (and possibly also H₂O) from an incompletely silylated glucuronic acid (*m/e* 258 corresponds to loss of CO₂ and two molecules of H₂O from a glucuronic acid substituted with two trimethylsilyloxy and two hydroxy groups). The mass spectra of the α -2-hydroxy lactone (X) and the β -5-hydroxy lactone (XI) were identical with those reported for compounds X5.2 and X5.3 respectively, apart from the varying contribution of the background peaks at *m/e* 258 and 243. The abundant C₂-C₅ ion

(V) R¹ = R² = R³ = trimethylsilyl

(VII) R¹ = H, R² = R³ = trimethylsilyl

(IX) R² = H, R¹ = R³ = trimethylsilyl

(XI) R³ = H, R¹ = R² = trimethylsilyl

(VI) R¹ = R² = R³ = trimethylsilyl

(VIII) R¹ = H, R² = R³ = trimethylsilyl

(X) R² = H, R¹ = R³ = trimethylsilyl

Scheme 1. Structures of various trimethylsilyl derivatives of glucuronolactone. G.l.c. and n.m.r. properties of these compounds are shown in Tables 1 and 2 respectively.

Table 1. Retention times R_f [relative to β -tris(trimethylsilyl)glucuronolactone] of various trimethylsilyl derivatives of glucuronolactone and glucuronic acid, compared with data of Kuenzle (1970b)

The incompletely silylated derivatives of glucuronolactone (shown in Scheme 1) and glucuronic acid were prepared as described in the Materials and Methods section.

Compound	3% SE-30, 155°C (Kuenzle, 1970b)	3% OV-101, 155°C	1% QF1, 175°C
Trace component (see the text)	0.50 (X5.1)	See text	
α -2-Hydroxy lactone (X)	0.60 (X5.2)	0.60	1.09*
β -5-Hydroxy lactone (XI)	0.70 (X5.3)	0.70	1.51
$\alpha\beta$ -1-Hydroxy lactone (VII)+ (VIII)	0.88 (X5.4)	0.88	1.73*
α -Tris(trimethyl- silyl) lactone (VI)	0.95	0.97	0.92
β -Tris(trimethyl- silyl) lactone (V)	1.00	1.00	1.00
β -2-Hydroxy lactone (IX)		1.00	1.43
Glucuronic acid incompletely silylated	1.47 (X5.5)	1.54	
Pentakis(trimethyl- silyl)gluco- furanuronic acid	1.68	1.77	
α -Pentakis(trimethyl- silyl)gluco- pyranuronic acid	2.20	2.40	
β -Pentakis(trimethyl- silyl)gluco- pyranuronic acid	2.98	3.40	

* Partial interconversion of α -2-hydroxy lactone and $\alpha\beta$ -1-hydroxy lactone occurs on 1% QF1, as seen by the increased level of the base line between R_f 1.09 and 1.73 on injection of a mixture of these compounds.

(m/e 158, shifted to m/e 159 on exchange with $^2\text{H}_2\text{O}$) is characteristic of the presence of a hydroxy group at C-2 or C-5, and corresponds to the ion $[\text{Me}_3\text{Si}-\text{O}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{O}-\text{SiMe}_3]^{++}$ (m/e 230) in the spectra of the tris(trimethylsilyl)lactones. Weak molecular ions M^{++} 320 (0.1–0.3% relative intensity, shifted to m/e 321 on exchange with $^2\text{H}_2\text{O}$) were not mentioned by Kuenzle (1970b). Instead peaks at m/e 377 (0.5% relative intensity) were reported, corresponding to ions $[M-\text{CH}_3]^+$ formed from completely silylated tris(trimethylsilyl)lactones. These ions were also observed in the spectra of monohydroxy lactones and could be due to background contributions (e.g. from prior runs), but more probably they are to be ascribed to intermolecular trans-silylation reactions on the hot metal surface of a jet separator. (Peaks at m/e 377 were

not observed when a silicone membrane separator was utilized.)

The mass spectra of compounds X5.4 and X5.5 were not reported, but their retention times (Table 2) correspond with those of the $\alpha\beta$ -1-hydroxy lactones (VII) and (VIII) and that of an incompletely silylated glucuronic acid. The more polar β -2-hydroxy lactone (IX) was co-eluted with β -tris(trimethylsilyl)glucuronolactone on 3% OV-101 and probably was overlooked by Kuenzle (1970b). It was easily purified by high-pressure liquid chromatography and separated on g.l.c. by using a 1% QF1 column (Table 1).

Starting from a 0.1M-HCl solution of glucuronic acid, evaporation and incomplete silylation yielded all compounds mentioned in Table 1, in proportions which were similar to those reported (Kuenzle, 1970b). The relative amount of β -5-hydroxy lactone, corresponding to the major component X5.3, was rather low for freshly prepared mixtures, but increased rapidly with time on storage of the silylated mixtures in dichloromethane solution at room temperature (22°C).

In conclusion, the present g.l.c.–mass spectrometry results show the identity of compounds X5.1 to X5.5 with incompletely silylated forms of glucuronolactone and glucuronic acid. N.m.r. and mass-spectral data support the structures shown in Scheme 1.

Discussion

An important result emerging from the present work is the identity of azopigments B₄, B₅ and B₆ (Kuenzle, 1970a,b) with the 2-, 3- and 4-*O*-acylglucuronide phenylazo compounds (II), (III) and (IV) (Compennolle *et al.*, 1978), as shown by t.l.c., mass spectrometry, and by the resistance to β -glucuronidase. It follows that either the disaccharide (Kuenzle, 1970b) or the glucuronide structural proposal (Compennolle *et al.*, 1970, 1978; Gordon *et al.*, 1976) for conjugated bilirubin-IX α is incorrect. The arguments supporting (a) the 1-*O*-acylglucuronide structures for the major conjugates of bilirubin-IX α and (b) their conversion into 2-, 3- and 4-*O*-acylglucuronides, form a consistent picture and will not be repeated here.

By contrast, the experimental basis for the disaccharide hypothesis shows several weaknesses, which are discussed below in the light of the new evidence. One argument for the disaccharide structures consisted of molecular-weight determinations based on weight/colour ratios. Contamination with non-pigment impurities such as silica (Kuenzle, 1970b) or bile acids (the present work) invalidates this approach.

Permethylation of the carbohydrates released from the azopigments with dilute ammonia failed to give the expected permethylated disaccharides,

Table 2. *N.m.r. spectra of various trimethylsilyl derivatives of glucuronolactone*

The preparation of the compounds (Scheme 1) and the conditions for the n.m.r. analysis are described in the Materials and Methods section. Spectra were measured in $[^2\text{H}]\text{chloroform}$ at 100 MHz.

Compound	Chemical shift (δ) (p.p.m.)						Coupling constant (Hz)					
	H ₁	H ₂	H ₃	H ₄ *	H ₅	OH	Si(CH ₃) ₃	J _{1,2}	J _{2,3}	J _{3,4}	J _{4,5}	J _{H,OH}
(V)	5.30	4.26	4.68	4.87	4.39		0.19, 0.22, 0.28	<0.5	< 0.5	4.5	6.2	
(VI)	5.45	4.20	4.70	4.84	4.47		0.21, 0.24, 0.285	4.0	~ 1.5	4.0	5.5	
(X)	5.63	4.28†	4.70‡	4.75‡	4.47	3.12†	0.25	4.5	< 0.5	~ 4‡	4.4	5.5†
(IX)	5.40	4.36§	4.82‡	4.90‡	4.40	2.01§	0.20, 0.28	< 0.5	< 0.5	~ 4‡	5.7	5.0§
(XI)	5.30	4.34	4.76	5.00	4.39		0.06, 0.24	< 0.5	< 0.5	5.2	7	
(VII)	5.35					2.88		< 0.5				
(VIII)	5.49¶					3.92¶		4.0				10

* H₄ appears as a doublet of doublets.

† Exchange of 2-OH protons is slow at room temperature (22°C). This results in the observation of a sharp doublet for the 2-OH proton and a doublet of doublets for H₂. Double-resonance experiments involving irradiation of H₁ and OH protons confirm the assignment of H₂ proton.

‡ H₃ and H₄ form the AB part of an ABX system (X=H₅).

§ H₂ and 2-OH protons appear as broad peaks at 22°C. At -20°C doublets are observed, which were assigned by irradiation of the 2-OH proton.

|| 1-OH and H₁ protons appear as broad peaks. The signal corresponding to H₁ collapses to a sharp singlet by irradiation of the 1-OH proton.

¶ 1-OH proton appears as a sharp doublet and H₁ proton as a doublet of doublets. The latter collapses to a doublet by irradiation of the 1-OH proton.

except for the sugar obtained from azopigment B₄. Even in the latter case, however, the yield of permethylated product was only 1%. Hence, the mixture of aldobiouronic acids detected by combined g.l.c.-mass spectrometry could well represent an artifact.

Acid hydrolysis of the carbohydrates obtained from azopigments B₄ and B₆ yielded mixtures of glucuronic acid and glucose. However, glucose probably was a contaminant (Gordon *et al.*, 1976), whereas the relative amount of glucuronic acid was under-estimated as a result of partial degradation in the acid medium (1M-HCl at 100°C for 1h). Finally, with the present work derivatives of the 'branched-chain' hexuronic acid, obtained from azopigment B₅ (Kuenzle, 1970b), were identified as incompletely silylated forms of glucuronolactone and glucuronic acid.

The evidence presented appears to leave no basis for the presence of carbohydrates other than glucuronic acid for conjugated azopigments B₄, B₅ and B₆. Certainly the two following claims cannot be justified (Kuenzle, 1970b, 1973): (a) trimethylsilylation of disaccharides, i.e. of conjugated azopigments and the carbohydrates released from them, removes the non-reducing terminal carbohydrate unit, giving rise to detection of only the reducing unit; (b) β -glucuronidase cleaves disaccharidic conjugates when the glucuronic acid moiety is attached to the pigment moiety (azopigments B₅ and B₆).

The present results relating to trimethylsilylation of the methyl esters of azopigments B₅ and B₆

again could be conceived as 'confirmation' of statement (a). However, the glucuronide structure has been established by n.m.r. and field-desorption mass spectrometry of the methyl ester of the ethyl anthranilate azopigment (I) and by electron-impact mass spectrometry of acetylated methyl esters of ethyl anthranilate azopigments (I), (II), (III) and (IV) (Compennolle *et al.*, 1978). Further, an exhaustive search failed to detect carbohydrates other than glucuronic acid for the purified azopigments (Gordon *et al.*, 1976; Compennolle *et al.*, 1978).

On treatment of azopigments B₄, B₅ and B₆ with β -glucuronidase we did not observe hydrolysis, in opposition to results for the phenylazo compound (I). No reaction was observed, either, for 2-, 3- and 4-O-acylglucuronide azopigments (Blanckaert *et al.*, 1978). The report that azopigments B₅ and B₆ were cleaved with β -glucuronidase (Kuenzle, 1970b) therefore appears puzzling. Although no controls involving inhibition of the enzyme with glucaro-1,4-lactone were performed, it seems likely that at least a part of the pigments analysed at that time had retained the 1-O-acylglucuronide structure. The absence of 1-O-acylglucuronide in the samples received by us could be due to (a) complete rearrangement of the bilirubin-IX α 1-O-acyl group during obstruction, collection and storage of bile and during chromatographic fractionation of bilirubin glucuronides or (b) rearrangement during storage of azopigment glucuronides. Rearrangement of azopigment glucuronides then would be restricted to the more readily occurring (1 $\rightarrow$ 2) acyl migration (Blanckaert

et al., 1978), as the progressive enrichment in the more polar components, found for azopigment mixtures B₅ and B₆, implies that equilibrium between all four positional isomers was not attained. Chromatography of the four positional isomers on reversed-phase columns (Compernelle *et al.*, 1978) showed that the 1-*O*-acylglucuronide was eluted with the more polar fractions of azopigments. This result is consistent with the 1-*O*-acylglucuronide being a component of the more polar azopigments B₅ and B₆ isolated in 1970 (Kuenzle, 1970a) as suggested by their cleavage with β -glucuronidase (Kuenzle, 1970b).

Separation of each of the azopigments B₄, B₅ and B₆ into three components by t.l.c. on polyamide sheets was reported by Kuenzle (1975, 1976, 1977). The mobilities and relative proportions of the three components were very similar in each case. Whether the latter result reflects formation of an equilibrium mixture of positional isomers or a separation of anomers and/or ring isomers cannot be decided from the evidence presented.

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