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Mass-Spectrometric Structure Elucidation of Dog Bile Azopigments as the Acyl Glycosides of Glucopyranose and Xylopyranose

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1. The structures of the α_2- and α_3-azopigments, prepared by diazotization of dog bile with ethyl anthranilate, were shown by mass spectrometry and g.l.c. to correspond to azobilirubin β-D-xylopyranoside and azobilirubin β-D-glucopyranoside respectively. 2. Both azopigments consist of a mixture of two methyl vinyl isomers having structures (IIIa) and (IIIb) for the α_2-azopigment and structures (IVa) and (IVb) for the α_3-azopigment. Separation of methyl vinyl isomers was obtained by t.l.c. or column chromatography performed on the acetylated azopigments. Hydrolysis of the less polar acetates derived from components (IIIa) and (IVa) gave rise to the azopigment (Ia), whereas hydrolysis of the more polar acetates derived from components (IIIb) and (IVb) gave rise to the azopigment acid (Ib). The positions of methyl and vinyl substituents in compounds (Ia) and (Ib) were assigned on the basis of their n.m.r. spectra. 3. Molecular ions in the mass spectra of the trimethylsilyl and acetyl derivatives of the azopigments indicated the presence of a pentose and a hexose conjugating sugar. 4. The ester functions linking the sugars to the propionic acid side chain of azobilirubin were demonstrated by ammonolysis and identification of the amide of azobilirubin as the aglycone derivative. 5. The sugar moieties were shown to occur as xylopyranose (α_2) and glucopyranose (α_3), bound at C-1, by application of a sequence of reactions performed on a micro-scale. The sugar hydroxyl groups were acetylated and the 1-acyl aglycone removed selectively by treatment with hydrogen bromide in acetic acid. Hydrolysis of the 1-bromo sugar acetates followed by acetylation afforded the α- and β-xylopyranose tetra-acetates and α- and β-glucopyranose penta-acetates, identified by a combination of g.l.c. and mass spectrometry. 6. The validity of this degradation scheme was confirmed (*a*) by g.l.c.–mass spectrometry identification of the α- and β-1-propionyl derivatives of glucopyranose tetra-acetate, obtained from the α_3-azopigment after final reaction with propionic anhydride; (*b*) by subjecting the acetates of $\alpha\beta$-glucopyranose, $\alpha\beta$-xylofuranose and $\alpha\beta$-glucofuranose to the same sequence of reactions.

The value of mass spectrometry for structure elucidation of bilirubin conjugates has recently been demonstrated for a glucuronic acid glycoside isolated from rat bile treated with diazotized ethyl anthranilate (Compernolle, Jansen & Heirwegh, 1970). By successive treatment with diazomethane and bistrimethylsilylacetamide, the glucuronic acid moiety of the azobilirubin was suitably protected to obtain a sufficiently volatile derivative. The great relative abundance of molecular ions in the mass spectrum of the latter contrasts with the spectra of trimethylsilyl derivatives of mono- and di-saccharides, and should be attributed to charge stabilization by the aromatic dipyrrole moiety. A similar charge-stabilizing effect was found for a 3-methyl-1-naphthyl derivative of maltose hepta-acetate by Karliner (1968).

A different approach was used by Kuenzle (1970) for characterization of disaccharidic conjugates of phenylazobilirubin: after hydrolysis of the glycosides in dilute ammonia solution, the free sugars were converted into fully methylated derivatives or subjected to acid hydrolysis followed by trimethylsilylation.

In the present work it is shown that the α_2- and α_3-azopigments derived from dog bile (Fevery, Van Hees, Leroy, Compernolle & Heirwegh, 1971) can be converted into derivatives suitable for mass spectrometry either by trimethylsilylation or by acetylation. Further, the acetylation procedure

blocks the ring configuration of the sugar moieties and, after specific removal of the 1-acyl aglycone, allows the assignment of xylopyranoside and glucopyranoside ring structures to the azopigments α_2 and α_3 respectively.

RESULTS AND DISCUSSION

The trimethylsilyl derivatives of α_2- and α_3-azopigments showed abundant molecular ions (Table 1) corresponding to azobilirubin conjugated with a pentose and a hexose sugar respectively. Substitution of all sugar hydroxyl groups gave rise to molecular ions at *m/e* 810 (azopigment α_2) and 912 (azopigment α_3), but either incomplete reaction or partial hydrolysis was indicated by less-abundant molecular ions at *m/e* 738 (azopigment α_2) and 840 (azopigment α_3). Even prolonged treatment with bistrimethylsilylacetamide in chloroform or with *N*-trimethylsilylimidazole in pyridine apparently failed to complete substitution. Partial hydrolysis of derivatives could occur, however, during introduction of the sample into the mass spectrometer and evaporation of reagents in the direct-introduction lock. Incomplete substitution has also been observed in the spectrum of a trimethylsilyl derivative of the δ-azopigment prepared from rat bile (Compernolle *et al.* 1970).

Other prominent ions in the spectra of trimethylsilyl derivatives of the α_2- and α_3-azopigments, i.e. *m/e* 165 and 119, are derived from the anthranilic part of the molecule (Compernolle *et al.* 1970). No other details of the structure of the sugar moieties could be derived owing to lack of suitable reference spectra and to possible interference of contaminating substances produced from the reagents.

Treatment with acetic anhydride–pyridine converted the azopigments α_2 and α_3 into the corresponding acetates (V) and (VI) (Scheme 1). Molecular ions found at *m/e* 720 (azopigment α_2) and 792 (azopigment α_3) confirmed that all hydroxyl groups of the sugar moieties had been substituted (Table 1). By t.l.c. both compounds (V) and (VI) were shown to consist of two closely moving components. That a separation of the respective methyl vinyl isomers (Va) and (Vb) and (VIa) and (VIb) had occurred was demonstrated by conversion of the single components of the acetates (V) and (VI), separated by t.l.c. or column chromatography, into the free acids (Ia) and (Ib). The hydrolysis was performed by application of the azopigment derivatives on a t.l.c. plate and treatment with 0.1M-sodium hydroxide; this procedure was more efficient than dissolution in mixtures of ammonia–water–methanol or 0.1M-sodium hydroxide–acetone. T.l.c. separation of compounds (Ia) and (Ib) as described by Compernolle *et al.* (1970) demonstrated that the less polar components of compounds (V) and (VI) had been converted into the less polar component of azopigment acid (I), whereas the more polar components of compounds (V) and (VI) were converted into the more polar component of azopigment acid (I).

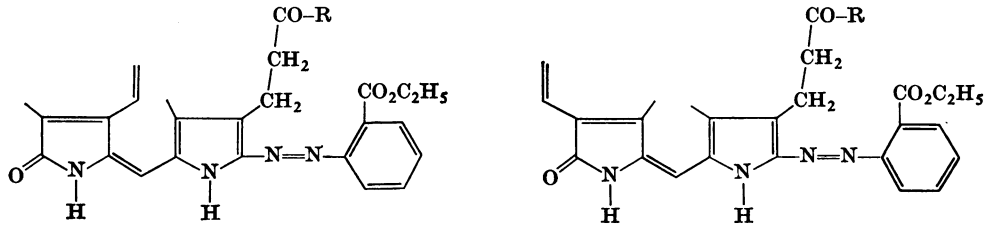
Finally, the dipyrrole parts of structures (Ia), (Va) and (VIa) were assigned to the less polar components and the dipyrrole parts of structures (Ib), (Vb) and (VIb) to the more polar components on the basis of the n.m.r. spectra of compounds (Ia) and (Ib). The n.m.r. spectrum of compound (Ia) showed signals for two methyl groups at δ 2.01 and 2.05, whereas for compound (Ib) two signals were found at δ 2.06 and 2.18. Clearly the signal for the methyl group in β-position of the carbonyl function in compound (Ib) is shifted downfield with respect to the methyl group in α-position of the carbonyl function in compound (Ia). Similarly signals for the vinyl group of compound (Ib) (δ 5.44–5.57 and 6.16–6.81) are centred upfield with respect to the signals for the vinyl group of compound (Ia) (δ 5.67–5.85 and 6.68–7.10).

After ammonolysis of the azopigments α_2 and α_3, xylose and glucose were isolated and identified by chromatographic techniques (Fevery *et al.* 1971). Mass spectra of their trimethylsilyl derivatives were identical with the spectra of authentic trimethylsilyl-xylose and trimethylsilyl-glucose

Table 1. *Molecular ions found in the mass spectra of trimethylsilyl and acetyl derivatives of azopigments α_2 and α_3*

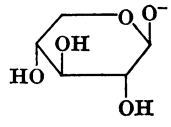
The third column gives the numbers of substituting groups introduced into the sugar moieties. The percentages in the fourth column reflect the degree of substitution in various experiments and were obtained from relative abundances of molecular ions.

Compound	Molecular ion (M^+)	No. of substituting groups	Percentage
Trimethylsilyl-azopigment α_2	738	2	10–30
	810	3	90–70
Trimethylsilyl-azopigment α_3	840	3	20–40
	912	4	80–60
Azopigment α_2 acetate	720	3	100
Azopigment α_2 acetate	792	4	100

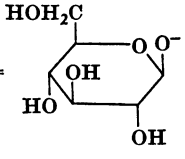


(Ia) R = OH (Ib)

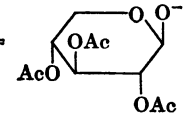
(IIa) R = NH_2 (IIb)



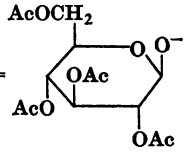
(IIIa) R = (IIIb)



(IVa) R = (IVb)



(Va) R = (Vb)

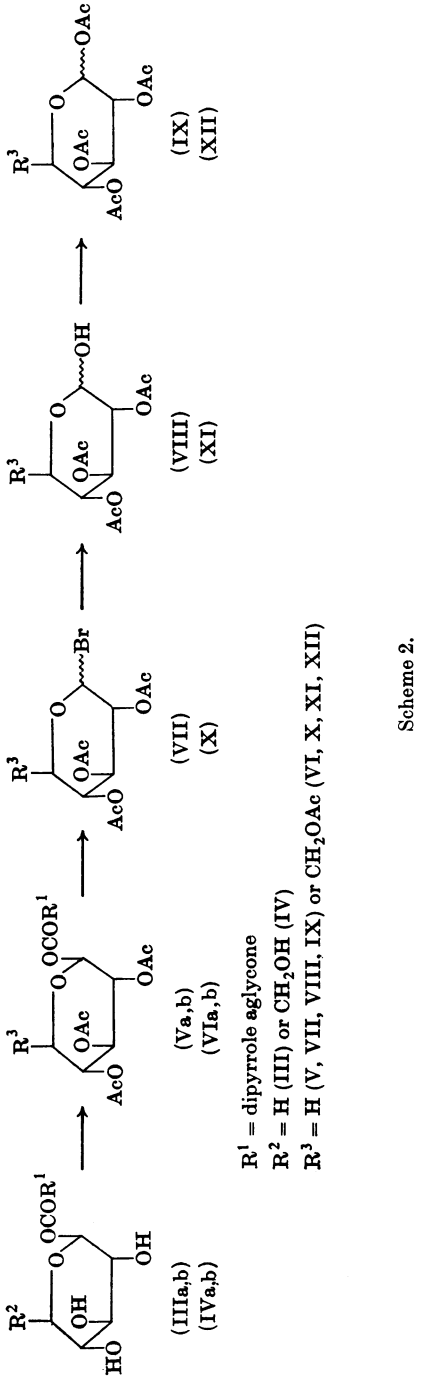


(VIa) R = (VIb)

Scheme 1.

respectively. The aglycones, isolated as the carboxylic acid amide of azobilirubin (compounds IIa and b) showed mass spectra (M^+ 461) identical with the one published by Compernolle *et al.* (1970). The isolation of compounds (IIa and IIb) proves the original existence of an ester linkage between the sugars and azobilirubin since, as mentioned by Compernolle *et al.* (1970), the carboxylic acid group of free azobilirubin (Ia,b) remains unchanged under the same experimental conditions.

The β-C-1 attachment of the aglycones to the sugars was demonstrated by enzymic studies (Fevery *et al.* 1971). The only problem left concerned the ring structures of the sugar moieties, because the ammonolysis reaction does not allow a differentiation between pyranoses and furanoses, since rapid equilibrium forms in alkaline medium between the various structures of free sugars. The acetates of the α_2- and α_3-azopigments appeared promising in this respect, because ring structures of the sugars are blocked by protection of the hydroxyl groups. Unfortunately, specific hydrolysis of the 1-acyl aglycone by dilute ammonia solution (Kuenzle, 1970) was not obtained. However, treatment with hydrogen bromide in acetic acid removed the 1-acyl aglycone in a specific way, giving rise to the 1-bromo derivatives of the xylose and glucose acetates (VII) and (X) (Scheme 2). The latter were converted into the corresponding 1-hydroxy derivatives (VIII) and (XI) by treatment with aqueous sodium hydrogen carbonate. Final acetylation led to formation of the α- and β-xylopyranose tetra-acetates (IX) and α- and β-glucopyranose penta-acetates (XII). Compounds (IX)



Scheme 2.

and (XII) were identified by direct comparison with the authentic materials by using the g.l.c.–mass spectrometry technique. The mass spectra of acetates of furanosides and pyranosides can be differentiated unequivocally, as shown by Biemann, De Jongh & Schnoes (1963) for the acetates of glucopyranose, ribopyranose, galactopyranose and galactofuranose. The spectra (Fig. 1*a*) obtained for the anomers of compound (IX) showed great analogy with those described for α- and β-ribopyranose tetra-acetate and were identical with the spectra of authentic α- and β-xylopyranose tetra-acetate. The spectra (Fig. 1*b*) of compound (XII) were identical with those described for α- and β-glucopyranose penta-acetate.

Two main degradation paths characterize the spectra (Fig. 1) of glucopyranose and xylopyranose acetates. Loss of the 1-acetoxy group [*m/e* 259 for compound (IX) and *m/e* 331 for compound (XII)] initiates consecutive eliminations of acetic acid and keten (path A). Sequence ions formed along path B require elimination of C-1 plus the ether oxygen together with one molecule of acetic acid [*m/e* 170 for compound (IX) and *m/e* 242 for compound (XII)] followed by losses of keten and acetic acid.

In analogy with the mass spectrum of β-D-galactofuranose penta-acetate published by Biemann *et al.* (1963), the spectra (Fig. 2) of α- and β-D-xylofuranose tetra-acetate and α- and β-D-glucofuranose penta-acetate (for synthesis see below) show prominent ions due to cleavage of the C-4–C-5 bond, i.e. *m/e* 245, 203 and 143 for the C-1–C-4 fragments, *m/e* 145 for C-5–C-6 and *m/e* 73 for C-5.

The α- and β-forms of compounds (IX) and (XII) could be identified by comparison of their retention times on g.l.c. (Tables 2 and 3) with the authentic materials and also by the greater relative abundances of the $M^+ - OAc$ ions in the spectra of the α-anomers (Biemann *et al.* 1963). The αβ mixtures of the intermediate glucose derivatives (X) and (XI) were analysed without prior g.l.c. separation of the anomeric forms. In the mass spectrum of compound (X) elimination of the reactive 1-bromo group is highly favoured, and hence fragmentation path A is found almost exclusively (ions at *m/e* 331, 271, 211, 169 and 109). On the other hand, the much less favourable loss of a 1-hydroxyl radical in compound (XI) results in the predominant formation of ions along path B (ions at *m/e* 240, 200, 140 and 98).

There remained one major problem to be solved, i.e. the validity of the degradation sequence (Scheme 2), utilized to establish the pyranoside structures for compounds (III) and (IV). Preferential cleavage of 1-acyl groups with hydrogen bromide in acetic acid does occur without interconversion of pyranosides and furanosides, if the pentoses and hexoses

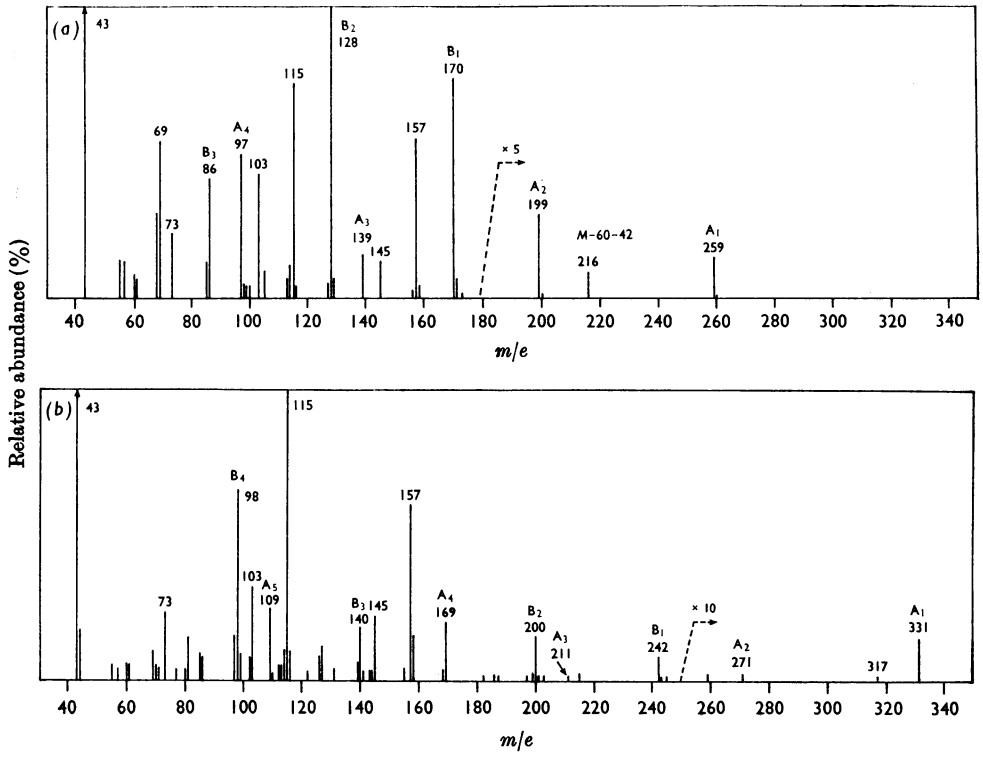


Fig. 1. (*a*) Mass spectrum of α-D-xylopyranose tetra-acetate obtained from the α_2-azopigment by application of the reaction sequence depicted in Scheme 2, followed by g.l.c.–mass spectrometry identification; (*b*) mass spectrum of α-D-glucopyranose penta-acetate obtained from the α_3-azopigment. Fragmentation path A refers to loss of the 1-acetoxy group from the molecular ion; path B refers to loss of C-1 plus the ether oxygen and acetic acid. Both reaction paths A and B are characterized by consecutive losses of acetic acid (60 mass units) or keten (42 mass units) and are indicated by the series A_1, A_2 etc. and by the series B_1, B_2 etc.

are protected as the more stable benzoyl derivatives (Fletcher, 1953; Ness, Fletcher & Hudson, 1951). However, Koenigs & Knorr (1901) found that tetra-acetyl-α-D-glucopyranosyl bromide reacted with methanol to give β-D-glucopyranose, and deacetylation also was observed in the reaction of triacetyl-α-L-rhamnopyranosyl bromide with methanol (Ness *et al.* 1951). In these reactions the hydrogen bromide liberated by methanolysis gives rise to an acid medium, in contrast with the slightly alkaline reaction conditions utilized in the present work.

One approach confirming the validity of the hydrolysis step performed on compounds (VII) and (X), consisted in a final treatment of the αβ-1-hydroxy derivatives (XI) with propionic anhydride instead of acetic anhydride. If any acetate group had been hydrolysed by treatment with aqueous sodium hydrogen carbonate, its replacement by a propionyl group would result in a corresponding mass shift for the ions formed along paths A and B. In fact the αβ-1-propionyl derivatives of compound (XI) were identified as the only reaction products derived from glucose by a comparison of the results of g.l.c.-mass spectrometry with the results obtained for compound (XII). Whereas compound (XII) and its 1-propionyl analogues showed different retention times, their mass spectra were very similar, as could be expected since the 1-substituents are lost in nearly all fragment ions.

The rates of cleavage of compounds (V) and (VI) by hydrogen bromide–acetic acid could not be determined by g.l.c. analysis, since any uncleaved material of high molecular weight would not be detected. This rate was estimated by allowing αβ-D-glucopyranose penta-acetate to react at room temperature for 60 min with hydrogen bromide–acetic acid (2:3, w/w) and quenching the reaction with bicarbonate solution. After final treatment with propionic anhydride–pyridine, g.l.c. analysis showed αβ-1-propionyl-D-glucopyranose tetra-acetate to be admixed with about 20% of starting material. When after 60 min reaction the hydrogen

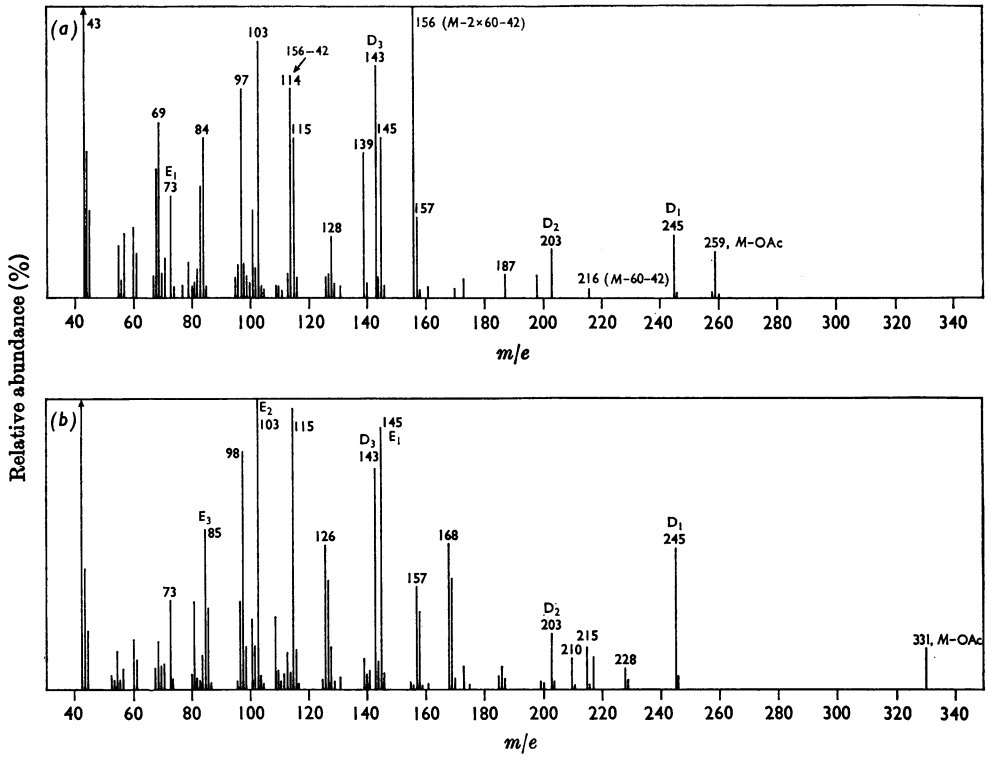


Fig. 2. (*a*) Mass spectrum of (α or β)-D-xylofuranose tetra-acetate (retention time 1.46 on 3% QF1; Table 2), obtained by g.l.c.–mass spectrometry analysis; (*b*) mass spectrum of (α or β)-D-glucofuranose penta-acetate (retention time 1.38 on 3% QF1; Table 3). Cleavage of the C-4–C-5 bond is referred to by paths D (charge retention on the C-1–C-4 fragments) and E (charge retention on C-5 or C-5–C-6). Consecutive losses of acetic acid (60 mass units) or keten (42 mass units) are indicated by the series D_1, D_2 etc. and by the series E_1, E_2 etc.

Table 2. *Relative retention times of acetates of xylose on a* 3% *QF*1 *column operated at* 160°C

Compound	Relative retention time
α-D-Xylopyranose tetra-acetate*†	1.00
β-D-Xylopyranose tetra-acetate*†	1.13
(α or β)-D-Xylofuranose tetra-acetate†	1.25
(β or α)-D-Xylofuranose tetra-acetate†	1.46
(α or β)-1-Propionyl-D-xylofuranose triacetate†	1.57
(β or α)-1-Propionyl-D-xylofuranose triacetate†	1.85

* Product derived from azopigment α_2.
† Synthetic product.

bromide–acetic acid solution was not quenched with bicarbonate solution but was evaporated at 60°C *in vacuo*, the proportion of products rose to 97:3. The possibility of αβ-D-glucopyranose penta-acetate arising from back exchange of the 1-bromo substituent with an acetoxy group in the hydrogen bromide–acetic acid solution is very unlikely because no compound (XII) was found after conversion of compound (VI) into the αβ-1-propionyl analogues of compound (XII).

In another approach to establish the validity of the degradation depicted in Scheme 2 αβ-D-xylofuranose tetra-acetate and αβ-D-glucofuranose penta-acetate were synthesized and subjected to the same sequence of reactions. Indeed, if any interconversion occurs, it should be in the direction: xylofuranosides and glucofuranosides → xylopyranosides and glucopyranosides. A synthesis of xylofuranose tetra-acetate, starting from xylose diethyl dithioacetal, has been described by Chang &

Table 3. *Relative retention times of acetates of glucose*

Compound	Relative retention time: 3% QF1, 180°C	Relative retention time: 1% JXR, 155°C
β-D-Glucopyranose penta-acetate*†	1.00	1.00
α-D-Glucopyranose penta-acetate*†	1.16	1.00
(α or β)-D-Glucofuranose penta-acetate†	1.16	
(β or α)-D-Glucofuranose penta-acetate†	1.38	
(α and β)-1-Propionyl-D-glucopyranose tetra-acetate†		1.38
(α or β)-1-Propionyl-D-glucofuranose tetra-acetate†	1.62	

* Product derived from azopigment α_3.
† Synthetic product.

Lythgoe (1950). Glucofuranose pentabenzoate was synthesized by Brigl & Gruener (1933) and by Reist, Spencer & Baker (1958).

By using methods outlined by Bishop & Cooper (1963) and Smirnyagin, Bishop & Cooper (1965), α-D-xylose and α-D-glucose were treated with methanolic hydrogen chloride to give mixtures of α- and β-methyl D-xylofuranoside and the four methyl D-glucosides respectively. These mixtures were subjected to consecutive treatments with acetic anhydride–pyridine, hydrogen bromide–acetic acid, aqueous sodium hydrogen carbonate and acetic anhydride–pyridine. Comparative g.l.c.–mass spectrometry analysis of the acetylated methyl glycosides and the final reaction mixtures showed the methyl furanosides to have reacted quantitatively with predominant formation of the expected furanose acetates. Some g.l.c. peaks, corresponding to substitution of furanose acetates with a bromine atom in one of the C-2–C-5 or C-2–C-6 positions were also detected. The acetylated αβ-methyl D-glucopyranoside mainly remained unchanged under the reaction conditions used. These observations are in line with the known greater reactivities of furanosides relative to pyranosides.

Fractions containing the α- and β-xylofuranose tetra-acetates and α- or β-glucofuranose penta-acetate were collected by preparative g.l.c. The latter g.l.c. fractions were subjected to the degradation described in Scheme 2. By final acetylation αβ-xylofuranose tetra-acetate and αβ-glucofuranose penta-acetate were obtained, again with no g.l.c. trace of pyranoside acetates. Final treatment with propionic anhydride–pyridine gave rise to the 1-propionyl derivatives of xylofuranose tetra-acetate and glucofuranose penta-acetate, admixed with unchanged furanose acetates.

EXPERIMENTAL

Materials

Azobilirubin (I) was separated into components (Ia) and (Ib) by t.l.c. as described by Compernolle *et al.* (1970). Larger quantities could be separated by using a modified t.l.c. system described below. Azopigments α_2 and α_3 were prepared from dog bile as described by Fevery *et al.* (1971). D-Glucose and D-xylose were purchased from E. Merck A.-G. (Darmstadt, Germany). Bistrimethylsilylacetamide and *N*-trimethylsilylimidazole were purchased from Pierce Chemical Co. (Rockford, Ill., U.S.A.).

Apparatus

Mass spectra. Spectra were recorded with an AEI MS12 mass spectrometer, electron energy 70eV, ionizing current 500μA and accelerating voltage 8kV. Trimethylsilyl derivatives of azopigments α_2 and α_3, acetates (V) and (VI) and amide (II) were introduced through the direct-introduction lock at ion-source temperatures slowly rising from 180° to 220°C. Trimethylsilyl derivatives of the isolated sugars and authentic xylose and glucose were run with the direct-introduction system, at an ion-source temperature of 100°C. The same experimental conditions were used for determining spectra of compounds (X) and (XI).

G.l.c.–mass spectrometry. A Pye series 104 chromatograph was used under the following conditions: glass column, 1.5m long, 6mm outside diameter; liquid phase, 3% QF1 or 1% JXR on Chromosorb W; carrier gas, He, 60ml/min. A 1:2 splitter divided the outlet of the column between respectively a flame ionization detector and the MS 12 mass spectrometer. A membrane separator (type V-5620 purchased from Varian, Palo Alto, Calif., U.S.A.), run at 200°C, allowed eluted substances to flow into the ion source, operated at 220°C. Very similar peak shapes were obtained by registration of the signals derived from the flame-ionization detector or from part of the total ion current of the mass spectrometer. Samples were injected directly on to the column, the operating temperatures of which are mentioned below.

N.m.r. spectra. Spectra were recorded at 100MHz in 0.04M solutions with a Varian HA-100 spectrometer in [$^2H_6$]dimethyl sulphoxide. Chemical shifts are given in p.p.m. relative to tetramethylsilane = 0 (internal standard). The spectrum of compound (Ia) showed signals at: δ 1.36, triplet $|J|$ 6Hz, 3H (CH_3 of ethyl group); δ 2.01, singlet, 3H (CH_3 group); δ 2.05, singlet, 3H (CH_3 group); δ 2.7, multiplet, 4H (CH_2–CH_2 of propionic acid side chain); δ 4.46, quadruplet, $|J|$ 6Hz, 2H (CH_2 of ethyl group); δ 5.67/5.77/5.85/6.68/6.80/6.86/6.97/7.03/7.10, multiplet, 3H (CH=CH_2 group); δ 6.16, singlet, 1H (=CH– methylene bridge); δ 7.3–7.9, multiplet 4H (aromatic protons of *o*-ethyloxycarbonyl-phenylazo

group). The spectrum of compound (Ib) showed signals at the same δ-values, except for δ 2.06 and 2.18, singlets, 3H (CH_3 groups); δ 5.44/5.47/5.54/5.57/6.33/6.37/6.52/6.62/6.70/6.81, multiplet (CH=CH_2 group); δ 6.14, singlet, 1H (=CH– methylene bridge).

Methods

Trimethylsilyl derivatives of azopigments α_2 and α_3. Azopigments α_2 and α_3 (1mg) were treated with 0.5ml of chloroform and a few drops of bistrimethylsilylacetamide. Alternatively azopigments α_2 and α_3 (1mg) were treated with 0.1ml of *N*-trimethylsilylimidazole–pyridine (1:3, v/v). The reactions were allowed to proceed for 5min, 1h and 16h, the solvents were evaporated *in vacuo* and a sample was introduced into the direct-introduction lock of the mass spectrometer, where the excess of reagent was evaporated off.

Trimethylsilyl derivatives of the sugars prepared from the azopigments α_2 and α_3. Purified freeze-dried sugars (0.1–0.5mg) obtained by ammonolysis of azopigments α_2 and α_3 (Fevery *et al.* 1971) were treated with 0.1ml of pyridine and 30μl of bistrimethylsilylacetamide. The mass spectra obtained were identical with those of the same derivatives of authentic xylose and glucose, after equilibration in dilute ammonia solution and freeze-drying.

Acetates of azopigments α_2 and α_3 (V) and (VI). Azopigments α_2 and α_3 (1mg) were dissolved in 0.3ml of pyridine–acetic anhydride (2:1, v/v) and allowed to react at room temperature for 4h. Ice and water were added and the solution was extracted with chloroform. The chloroform layer was washed successively with $NaHCO_3$ solution until acid-free and then with water. After evaporation of chloroform *in vacuo*, the last traces of pyridine were removed by three additions of benzene followed by evaporation *in vacuo*.

T.l.c. separations of compound (V) into components (Va) and (Vb) and of compound (VI) into components (VIa) and (VIb) were performed by application of compounds (V) and (VI) as narrow bands on pre-coated silica-gel plates (Merck F254) and development with the solvent system benzene–ethyl acetate (17:3, v/v). Collected silica-gel fractions were eluted with pentan-2-one. Alternatively compound (VI) was separated into components (VIa) and (VIb) by column chromatography (silica gel 0.05–0.2mm; E. Merck A.-G.). The pigment, adsorbed on a little silica gel, was applied on to a column (60cm×3.5cm) of silica-gel packed in benzene. Components (VIa) and (VIb) separated after elution for 2 days with benzene–ethyl acetate (19:1, v/v). The appropriate silica-gel fractions were extruded and extracted with pentan-2-one.

Conversion of compounds (Va) and (Vb) and compounds (VIa) and (VIb) into compounds (Ia) and (Ib). The single compounds (Va), (Vb), (VIa) and (VIb) were applied to pre-coated silica-gel plates (DC-Alufolien; E. Merck A.-G.). Then 0.1M-NaOH (0.5ml) was allowed to infuse slowly (20min) into the coloured zone of silica gel. After 30min 0.1M-acetic acid (0.5ml) was applied and allowed to evaporate. After elution of collected silica-gel fractions with pentan-2-one, the azopigments (Ia) and (Ib) were examined by t.l.c. on pre-coated silica-gel plates (Merck F254) in the solvent system benzene–ethyl acetate (1:1, v/v).

Instead of the time-consuming procedure with repetitive developments described by Compernolle *et al.* (1970), the solvent was eluted from the top of the plate into a piece of Whatman no. 3 paper (20cm×15cm), fixed to the upper end of the plate with joiner's glue and folded on the back of the plate. In this way conversion of the less polar azopigment acetates (Va) and (VIa) into the less polar azopigment acid (Ia) was established, whereas the more polar compounds (Vb) and (VIb) were converted into the more polar acid (Ib).

Xylopyranose tetra-acetate (IX) from the triacetate of α_2-azopigment. Compound (V) [0.2mg; mixture of components (Va) and (Vb)] was allowed to react for 1h at room temperature with 0.3ml of hydrogen bromide–acetic acid (2:3, w/w). The solution was evaporated *in vacuo* (bath temperature 40°C) to give a residue containing compound (VII). $NaHCO_3$ (1g), water (3ml) and acetone (3ml) were added and the solution was allowed to stand for 1h. After dilution with water to dissolve precipitated $NaHCO_3$, the solution was extracted with methylene chloride (3×3ml). Drying over Na_2SO_4 and evaporation *in vacuo* yielded a residue containing compound (VIII). This residue was treated with 0.2ml of acetic anhydride–pyridine (1:2, v/v) for 3h to give compound (IX). Reagents were removed by repeated additions of benzene, followed by evaporation *in vacuo*.

G.l.c.–mass spectrometry (Table 2) performed on compound (IX) with a 3% QF1 column operated at 160°C revealed a 2:3 mixture of α-xylopyranose tetra-acetate and β-xylopyranose tetra-acetate.

Reference materials were prepared from α-D-xylose as follows: treatment with acetic anhydride–pyridine at room temperature led to predominant formation of α-D-xylopyranose tetra-acetate; boiling of α-D-xylose in pyridine followed by addition of acetic anhydride gave rise to a mixture enriched in β-D-xylopyranose tetra-acetate.

Glucopyranose penta-acetate (XII) from the tetra-acetate of α_3-azopigment. Compound (VI) [0.2mg; mixture of components (VIa) and (VIb)] was converted into compound (XII) in exactly the same way as described for the preparation of compound (IX) from compound (V). Mass spectra were determined on crude intermediates (X) and (XI).

G.l.c.–mass spectrometry (Table 3), performed on compound (XII) by using a 3% QF1 column operated at 180°C, revealed the presence of a 7:3 mixture of α-D-glucopyranose penta-acetate and β-D glucopyranose penta-acetate. Reference materials, i.e. mixtures enriched in α- or β-glucopyranose penta-acetate, were prepared from α-D-glucose in the same way as described for the preparation of reference materials from xylose.

Treatment of compound (XI) with 0.2ml of propionic anhydride–pyridine (1:2, v/v) led to formation of αβ-1-propionyl-D-glucopyranose tetra-acetate. G.l.c.–mass spectrometry (Table 3) performed by using a 1% JXR column failed to separate the α- and β-anomers, but demonstrated the absence of any glucopyranose penta-acetate.

α- and β-D-Xylofuranose tetra-acetate. α-D-Xylose (20mg) was dissolved in 10ml of methanolic 0.2M-HCl. After 5h the reaction was quenched by addition of pyridine (5ml). Reagents were evaporated off *in vacuo*, and pyridine was removed by three additions of benzene followed by evaporation *in vacuo*. The residue was

allowed to react overnight with 2ml of pyridine–acetic anhydride (2:1, v/v). The reaction mixture was poured into cold saturated $NaHCO_3$, and the aqueous solution was extracted with methylene chloride. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. G.l.c.–mass-spectrometry analysis (3% QF1, 155°C) indicated nearly quantitative conversion into a 1:1 mixture of α- and β-methyl-D-xylofuranose triacetate. About 5% of α- and β-xylopyranose tetra-acetate, derived from unchanged α-D-xylose, was detected.

The crude αβ-methyl-D-xylofuranose triacetate was treated with 0.5ml of hydrogen bromide–acetic acid (2:3, w/w) for 1h. Reagents were evaporated *in vacuo* and to the residue $NaHCO_3$ (1g), water (3ml) and acetone (3ml) were added. After 1h the solution was diluted with water and extracted with methylene chloride. The methylene chloride layer was washed with water, dried over Na_2SO_4 and treated with 0.3ml of pyridine–acetic anhydride (2:1, v/v) for 3h. After evaporation *in vacuo* the reaction mixture was analysed by g.l.c.–mass spectrometry by using a 3% QF1 column operated at 160°C. The analysis showed α- and β-xylofuranose tetra-acetate to be the main reaction products, admixed with 5% of α- and β-xylopyranose tetra-acetate. In front of the four peaks corresponding to xylose tetra-acetates some materials were eluted whose mass spectra indicated furanose acetate structures substituted with a bromine atom in one of the C-2–C-5 positions. By collecting the appropriate g.l.c. fractions a mixture of α- and β-xylofuranose tetra-acetate (0.1mg) was obtained.

α- and β-D-Glucofuranose penta-acetate. Essentially the same procedure as described for the preparation of xylofuranose tetra-acetate was utilized. α-D-Glucose (20mg) was treated with 10ml of methanolic 2M-HCl for 2h. G.l.c.–mass-spectrometry analysis (3% QF1, 160°C) of the acetylated methyl glucosides revealed the presence of a 3:2 mixture of methyl furanosides and methyl pyranosides, admixed with 4% of α- and β-glucopyranose penta-acetate (derived from unchanged D-glucose).

G.l.c.–mass spectrometry analysis (3% QF1, 180°C) of the glucose penta-acetates, obtained by applying the same reaction sequence as described for the preparation of xylofuranose tetra-acetate, indicated quantitative conversion of the acetylated methyl glucofuranosides. Again the main reaction products α- and β-glucofuranose penta-acetate were admixed with furanose acetates substituted with a bromine atom in one of the C-2–C-6 positions. In contrast a 2/1 ratio was found for unchanged αβ-methyl D-glucopyranose tetra-acetate and its reaction product αβ-D-glucopyranose penta-acetate. The material (0.1mg) corresponding to α- or β-glucofuranose penta-acetate (retention time 1.38; Table 3) was collected by preparative g.l.c. (3% QF1, 180°C).

Application of reaction sequence to αβ-glucopyranose penta-acetate. A 15:1 mixture of α- and β-D-glucopyranose penta-acetate (10mg) was allowed to react with 0.5ml of hydrogen bromide–acetic acid (2:3, w/w). After 1h the reaction mixture was poured into a saturated $NaHCO_3$ solution. Alternatively the reaction mixture was evaporated *in vacuo* (bath temperature 60°C) before treatment with $NaHCO_3$ solution. After extraction with methylene chloride and treatment with propionic anhydride–pyridine (2:1, v/v), the reaction products were analysed by g.l.c.–mass spectrometry (1% JXR, 155°C). The analysis (Table 3) revealed the presence of 4:1 and 97:3 mixtures of αβ-1-propionyl-D-glucopyranose tetra-acetate and αβ-D-glucopyranose penta-acetate (prepared by treatment with $NaHCO_3$ and by evaporation at 60°C respectively as described above).

Application of reaction sequence to αβ-D-xylofuranose tetra-acetate. The material (0.1mg) obtained by preparative g.l.c. was dissolved in 0.3ml of methylene chloride and allowed to react for 1h with 0.3ml of hydrogen bromide–acetic acid (2:3, w/w). After preparation and treatment of the reaction mixture (evaporation *in vacuo* at a bath temperature of 30°C and final acetylation), g.l.c.–mass spectrometry (3% QF1, 160°C) indicated the presence of only α- and β-xylofuranose tetra-acetate. By final treatment with propionic anhydride–pyridine a 7:3 mixture of αβ-1-propionyl-D-xylofuranose tri-acetate and αβ-D-xylofuranose tetra-acetate was obtained.

Application of reaction sequence to α- or β-D-glucofuranose penta-acetate. The material (0.1mg) obtained by preparative g.l.c. (retention time 1.38; Table 3) was treated in the same way as described for αβ-D-xylofuranose tetra-acetate. After acetylation, g.l.c.–mass spectrometry (3% QF1, 180°C) indicated the presence of only α- and β-D-glucofuranose penta-acetate. Alternative treatment with propionic anhydride–pyridine gave rise to a 4:1 mixture of αβ-1-propionyl-D-glucofuranose tetra-acetate and αβ-D-glucofuranose penta-acetate.

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