The Preparation and Characterization of Bile Pigments

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Brief reduction of bilirubin with dilute sodium amalgam was shown to give chromogens containing both vinyl and ethylidene β -substituents. Acid-catalysed rearrangement of these chromogens with bilirubin or mesobilirubin and subsequent dehydrogenation gave a range of new violins containing unconjugated ethylidene and vinyl substituents. Rearrangements between mesobilirubinogen, bilirubin and mesobilirubin gave dihydrobiliviolins, mesobiliviolins, biliverdins, dihydrobiliverdins and mesobiliverdins of the III α , IX α and XIII α series. In this way 30 compounds were prepared, purified by t.l.c. as dimethyl esters, and characterized by n.m.r., mass and electronic spectroscopy, and by chemical interconversion and degradation.

A major path of haemoglobin breakdown results in the formation of biliverdin, which is converted into bilirubin by tissue dehydrogenases. A variety of bilirubin conjugates are excreted in the bile into the intestine, where hydrolysis and a variety of sequential hydrogenations provides a series of chromogens which on dehydrogenation result in a variety of bile pigments. These include urobilins and violins of various stereochemistries, some of which may retain vinyl groups. There is need for pure samples of such substances to act as model compounds.

The products obtained by FeCl₃ dehydrogenation of the chromogens obtained by the reduction of bilirubin for 1 h with 3% sodium amalgam included bile pigments of the III α and XIII α as well as IX α isomer series (Stoll & Gray, 1970), and an acid-catalysed rearrangement of bilirubin has also been shown to lead to its III α and XIII α isomers (McDonagh & Assisi, 1971, 1972).

The present paper describes (a) the product of brief reduction of bilirubin with more dilute (<1%) sodium amalgam and (b) a general rearrangement

reaction of IX α bile pigments to III α and XIII α isomers. Some 30 pure bile pigments have been prepared and fully characterized by physicochemical techniques, and should be of value in the elucidation of the structure of the ever-increasing varieties of bile pigments occurring in natural products, especially as III α and XIII α compounds may arise during metabolism *in vivo*.

Nomenclature

The nomenclature of bile pigments presents many difficulties and an IUPAC–IUB committee has made recommendations which have not yet been published.

The recommendations for nomenclature have been given, with trivial names, in Table 1. The recommendations for the numbering of atoms are shown in the Tables and Figures of n.m.r. data. In the text we have used trivial names derived as follows.

In the unsymmetrical IX α compounds the four rings and the three bridge carbon atoms have been labelled thus:

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where X is ethyl, vinyl or ethylidene, and Y is ethyl or vinyl.

The two main groups of compounds discussed are

Table 1. *Nomenclature of pigments described in text*

Compound no.	Trivial name used in text	IUPAC-IUB semi-systematic name
1	Biliverdin III α	[3-Methyl]-[2-vinyl]-biliverdin
2	Biliverdin IX α	Biliverdin
3	Biliverdin XIII α	[18-Methyl]-[17-vinyl]-biliverdin
4	Dihydrobiliverdin III α	[2-Ethyl]-[3-methyl]-biliverdin
5	A-Dihydrobiliverdin IX α	[3-Ethyl]-biliverdin
6	D-Dihydrobiliverdin IX α	[18-Ethyl]-biliverdin
7	Dihydrobiliverdin XIII α	[17-Ethyl]-[18-methyl]-biliverdin
8	Mesobiliverdin III α	[2-Ethyl]-[3-methyl]-mesobiliverdin
9	Mesobiliverdin IX α	Mesobiliverdin
10	Mesobiliverdin XIII α	[17-Ethyl]-[18-methyl]-mesobiliverdin
11	Biliviolin III α	[3-Methyl]-[2-vinyl]-15,16-dihydrobiliverdin
12	Biliviolin IX α	15,16-Dihydrobiliverdin
13	'Exo'-dihydrobiliviolin III α	[2-Ethyl]-[3-methyl]-4,5-dihydrobiliverdin
14	'Endo'-dihydrobiliviolin III α	[2-Ethyl]-[3-methyl]-15,16-dihydrobiliverdin
15	A-Dihydroisobiliviolin IX α	[3-Ethyl]-4,5-dihydrobiliverdin
16	A-Dihydrobiliviolin IX α	[3-Ethyl]-15,16-dihydrobiliverdin
17	D-Dihydrobiliviolin IX α	[18-Ethyl]-15,16-dihydrobiliverdin
18	'Exo'-dihydrobiliviolin XIII α	[17-Ethyl]-[18-methyl]-15,16-dihydrobiliverdin
19	Mesobiliviolin III α	[2-Ethyl]-[3-methyl]-4,5-dihydromesobiliverdin
20	Isomesobiliviolin IX α	4,5-Dihydromesobiliverdin
21	Mesobiliviolin IX α	15,16-Dihydromesobiliverdin
22	Mesobiliviolin XIII α	[17-Ethyl]-[18-methyl]-4,5-dihydromesobiliverdin
23	'R*R*-Ethylidene'-dihydroisobiliviolin IX α	(2R*,4R*)-[3-Ethylidene]-2,3,4,5-tetrahydrobiliverdin
24	'R*R*-Ethylidene'-dihydrobiliviolin XIII α	(16R*,18R*)-[17-Ethylidene]-[18-methyl]-15,16,17,18-tetrahydrobiliverdin
25	'R*S*-Ethylidene'-dihydroisobiliviolin IX α	(2R*,4S*)-[3-Ethylidene]-2,3,4,5-tetrahydrobiliverdin
26	'R*S*-Ethylidene'-dihydrobiliviolin XIII α	(16R*,18S*)-[17-Ethylidene]-[18-methyl]-15,16,17,18-tetrahydrobiliverdin
27	'R*R*-Ethylidene'-isomesobiliviolin IX α	(2R*,4R*)-[3-Ethylidene]-2,3,4,5-tetrahydromesobiliverdin
28	'R*R*-Ethylidene'-mesobiliviolin XIII α	(16R*,18R*)-[17-Ethylidene]-[18-methyl]-15,16,17,18-tetrahydromesobiliverdin
29	'R*S*-Ethylidene'-isomesobiliviolin IX α	(2R*,4S*)-[3-Ethylidene]-2,3,4,5-tetrahydromesobiliverdin
30	'R*S*-Ethylidene'-mesobiliviolin XIII α	(16R*,18S*)-[17-Ethylidene]-[18-methyl]-15,16,17,18-tetrahydromesobiliverdin

(i) the verdins in which the whole skeleton as depicted above is conjugated, and (ii) the violins in which either the 5- or the 15-bridge of the verdin is hydrogenated.

(i) In the verdin group basic structures are biliverdins III α , IX α and XIII α , each containing two vinyl substituents. The prefix dihydro- implies hydrogenation of one vinyl group to an ethyl group, e.g. A-dihydrobiliverdin IX α bears an ethyl group in ring A and a vinyl group in ring D. In the symmetrical III α and XIII α series the distinction between A-ring and D-ring substitution disappears and there is thus only one III α and one XIII α dihydrobiliverdin. The prefix meso- implies hydrogenation of both vinyl groups to ethyl groups.

(ii) In the violin group the unsymmetrical chromophore introduces further complexity. Thus there are four basic structures, biliviolins III α , IX α and XIII α and isobiliviolin IX α . Biliviolin and isobiliviolin IX α differ in that the former has the D ring unconjugated

and the latter has the A ring unconjugated. The prefixes dihydro- and meso- have the same meanings as in the verdin series. A further complication arises in the name of dihydroviolins in the symmetrical III α and XIII α series because of the lack of distinction between the end rings on the basis of side-chain order. The prefixes 'exo-' and 'endo-' have been used to imply hydrogenation of the vinyl group outside the chromophore (in the unconjugated end ring) and of the vinyl group inside the chromophore (in the conjugated end ring) respectively. Finally, the naming of the new violins containing an ethylidene substituent in the unconjugated end ring has been done by using the prefixes 'R*R*-ethylidene-' and 'R*S*-ethylidene-' before the name of the violin into which the new compound is converted by base-catalysed isomerization of the ethylidene double bond.

The 'R*R*-ethylidene-' and 'R*S*-ethylidene-' violins are diastereoisomers having opposite relative configurations at the outer asymmetric carbon atom

in the 2- or 18- position bearing a secondary methyl substituent and the inner asymmetric carbon atom in the 4- or 16-position of the unconjugated end ring.

The term 'ethylidene'-violin refers to either an '*R***R*'- or an '*R***S*'-ethylidene'-violin where stereochemistry is not specified.

The term 'normal'-violin refers to any violin containing a pyrrolenone unconjugated end ring, and in the present study all violins, except 'ethylidene'-violins, are 'normal'-violins.

Experimental

Materials

Bilirubin, mesoporphyrin and protoporphyrin were from Sigma (London) Chemical Co., London S.W.6, U.K. BF_3 and NH_3 were from Cambrian Chemicals, Croydon, Surrey, U.K. 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone, 1,2-dichloroethane, dichloromethane, dichlorodimethylsilane, dried MgSO_4 , methyl acetate, methyl propionate and NaBH_4 were general-purpose reagent grade. Butan-2-one was special for chromatography. Aluminium oxide (Brockmann grade I, neutral) was used for column chromatography. Tetramethylsilane was n.m.r. reagent grade. Deuteriochloroform of 99.8% isotopic purity was from Prochem, London S.W.18, U.K. Chromosorb W and cyclohexanedimethanol succinate were from Perkin-Elmer, Beaconsfield, Bucks., U.K. Other reagents were analytical grade from commercial sources.

For t.l.c. 1,2-dichloroethane, dichloromethane and methyl propionate were purified by washing successively with aq. HCl, aq. NaHCO_3 and water, and then drying over MgSO_4 and distilling.

Silica gel D5, Chromatocharger and 0.1 ml Hamilton syringes were from Camag Chemie-Erzeugnisse und Adsorptionstechnik A.-G., Muttenz, Switzerland. T.l.c. apparatus was supplied by Shandon Scientific Co., London N.W.10, U.K.

Methylethylidenesuccinimide was a gift from Professor W. Rüdiger, University of Saarland, Saarbrücken, Germany, and synthetic mesobiliviolin IX α dimethyl ester and isomesobiliviolin IX α dimethyl ester were gifts from Professor H. Plieninger, University of Heidelberg, Heidelberg, Germany.

Thin-layer chromatography

The pigments were separated as dimethyl esters by chromatography on silica gel D5 (similar to silica gel G, but with higher resolution for these pigments). The solvent systems used were: (1) carbon tetrachloride / dichloromethane / methyl acetate / methyl propionate (2:2:1:1, by vol.); (2) 1,2-dichloroethane/butan-2-one (2:1, v/v); (3) benzene/ethanol (10:1, v/v); (4) carbon tetrachloride / dichloro-

methane/methyl acetate/methyl propionate (1:1:1:1, by vol.); (5) 1,2-dichloroethane/butan-2-one (1:1, v/v); (6) carbon tetrachloride / dichloromethane / methyl acetate/methyl propionate (1:1:2:2, by vol.); (7) 1,2-dichloroethane/butan-2-one (1:2, v/v).

Pigments were applied as thin streaks, with a Camag Chromatocharger, as a concentrated solution in chloroform to 20 cm $\times$ 20 cm plates coated to a depth of 0.3 mm. These were developed in the appropriate solvent system by single, double or triple development (see under 'Preparation of individual pigments'). If multiple development was used, the plates were removed from the development tank when the solvent reached the top of the plate and were dried with a warm-air blower, and replaced in the tank for subsequent development. When development was complete the plates were dried with a warm-air blower and the individual bands scraped off by suction. The pigment was washed on the silica gel with carbon tetrachloride several times to remove all traces of the development solvents. The pigments were then eluted with acetone and recovered by evaporation in a stream of O_2 -free N_2 at 40–50°C. Finally a small volume of carbon tetrachloride was added and evaporated, and this process repeated several times to remove all traces of acetone. In this way impurity signals in the n.m.r. spectra were minimized.

Reduction with sodium amalgam

Bilirubin (100 mg) was dissolved in a minimum volume of 0.1 M-NaOH (usually approx. 10 ml). Liquid sodium amalgam (0.5%) (Stoll & Gray, 1970) was added, a little at a time, with vigorous shaking. The solution rapidly became pale straw colour, and was ready for immediate use. It must neither be left in contact with excess of sodium amalgam, nor remain strongly alkaline.

Acid-catalysed rearrangement (coupling)

The method is a modification of that of McDonagh & Assisi (1972). Pigment (100 mg) was dissolved in dimethyl sulphoxide (20 ml), and O_2 -free N_2 was passed through the solution for 10 min. HCl (12 M; 1.25 ml) was then added dropwise while N_2 was still being passed. After about 1 min water (20 ml) was added to quench the reaction and precipitate the product. The mixture was then extracted exhaustively with chloroform, and the chloroform solution was dried by filtration through cottonwool and evaporated to dryness *in vacuo* at about 40°C to recover the product.

Dehydrogenation with FeCl_3

Pigment (100 mg) was dissolved in methanol (100 ml), and 20% (w/v) anhydrous FeCl_3 in 12 M-HCl

(10ml) was added. The mixture was refluxed for 30–90 min, depending on the extent of dehydrogenation required. The mixture was diluted with water, and the pigments were extracted into chloroform. The chloroform solution was washed with water to remove all acid and salts, dried by filtration and evaporated to dryness.

Dehydrogenation with 2,3-dichloro-5,6-dicyano-p-benzoquinone

(a) *For rubins.* Pigment (100mg) was dissolved in dimethyl sulphoxide (approx. 50ml), and a freshly prepared solution of 2,3-dichloro-5,6-dicyano-p-benzoquinone (approx. 100mg) in dimethyl sulphoxide (approx. 2ml) was added dropwise with constant stirring until no further colour change was observed; then acetic acid (approx. 5ml) was added.

(i) Free verdins were recovered by precipitation with saturated aqueous sodium acetate and filtration by suction through sintered glass. After being washed with water and suction-dried they were usually immediately esterified with methanolic BF_3 (100ml); for details, see below.

(ii) Free or esterified violins were recovered by addition of water and exhaustive extraction into chloroform. If free verdin was also present and not required this could be largely removed by repeated washing of the chloroform solution with water, when the verdin formed an interfacial precipitate. The chloroform solution of violin was then dried by filtration and evaporated to dryness.

(b) *For urobilinogens.* Freshly prepared urobilinogens in 0.1 M-NaOH (approx. 10ml) were mixed with methanol (200ml) under O_2 -free N_2 . Acetic acid (1M; approx. 1ml) was added with stirring, and, without delay, 2,3-dichloro-5,6-dicyano-p-benzoquinone (approx. 100mg) in methanol (approx. 2ml) was added dropwise with stirring until no further colour change was observed. Acetic acid (approx. 2ml) was added. The methanolic solution containing urobilin was used without further purification.

Hydrogenation in 0.1 M-NaOH of bilirubin to mesobilirubin (Fischer & Haberland, 1935)

Bilirubin (100mg) was dissolved in 0.1 M-NaOH (10ml), and 10% (w/w) palladium on charcoal (20mg) was added as a suspension in a small volume of 0.1 M-NaOH. Hydrogen was passed through the solution as a steady stream, fast enough to maintain the catalyst in suspension. The course of the hydrogenation was followed spectrophotometrically (see below), and, when complete, hydrogenation was continued for a few more minutes, after which the mixture was filtered to remove catalyst; the mesobilirubin was precipitated by dropwise addition of acetic acid with stirring. The precipitate was filtered at the pump on

sintered glass, washed with water and then with methanol. The latter removed substantial quantities of an unknown yellow pigment. The residue of mesobilirubin was dried and was of sufficient purity for further use.

Test for hydrogenation. One drop of the reaction mixture was added to methanol (approx. 5ml), and 1 drop of saturated methanolic zinc acetate was added. Methanolic iodine (0.1%) was then added dropwise until the orange solution became blue with a red fluorescence. One drop of methanolic 4M- NH_3 was then added, and the solution was compared in the Hartridge Reversion Spectroscope with solutions similarly prepared from pure bilirubin and mesobilirubin. The shift of the absorption line at 638nm derived from bilirubin, to 626nm derived from mesobilirubin, was used as the index of hydrogenation (Pearson & Watson, 1963).

Hydrogenation in formic acid (Ó Carra & Colleran, 1970)

The dimethyl ester of the pigment (approx. 100 μg) was dissolved in 98% formic acid (1ml). Palladium on charcoal (10%, w/w, 1–2mg) was added as a suspension in a few drops of formic acid. A slow stream of hydrogen (about 1 bubble/s) was passed through the solution. Biliverdins required 30–40min, and dihydroverdins and violins required 15–20min. The change of colour from green to blue for verdins and from blue-violet to deep violet for violins was used as a guide to the reaction. Water and chloroform were added and the chloroform layer containing pigment was separated and washed with water, 0.5 M- NaHCO_3 solution (with care to avoid loss by evolution of CO_2) and water. Finally it was dried by filtration and evaporated to dryness.

Reduction with NaBH_4

The pigment was dissolved in methanol, and solid NaBH_4 was added. The solution was warmed to about 50°C. The reaction was complete when the colour of the verdin or violin was changed completely to yellow. Chloroform was added and then water, and the chloroform layer of pigment was separated, washed several times with water, dried by filtration through chloroform-moistened paper and evaporated to dryness under N_2 .

Methyl esterification with methanolic BF_3 (modification of method of Cole et al., 1967)

(a) *For dry pigments soluble in cold methanol (violins).* The pigment was dissolved in methanol, and methanolic BF_3 was added to give a final concentration of 1% (w/w) BF_3 . The solution was left at about 22°C for 15h. Chloroform was added and

then water, and the chloroform layer of pigment quickly separated and washed with water, dilute aqueous sodium acetate and again with water, dried by filtration as above and evaporated to dryness.

(b) *For pigments soluble only in hot methanol (verdins)*. Pigment was dissolved in boiling methanol, and methanolic BF_3 was added to give 5% (w/w) BF_3 concentration. This solution was refluxed for 10 min. After cooling, the pigments were extracted as in (a) above. Moist pigment dried only by suction on the pump may be used in this preparation, provided that at least 100 ml of methanol is used per 100 mg of pigment.

Isomerization with sodium methoxide

Pigment was dissolved in 0.25 M-sodium methoxide (from sodium and methanol) in methanol under O_2 -free N_2 . After standing in the dark (for time see below) a slight excess of 0.25 M-acetic acid was added and the pigment extracted into chloroform. The chloroform solution was washed with water, dried by filtration and evaporated to dryness in a stream of the N_2 .

'R*R*-Ethylidene'-violins were maximally converted into 'R*S*-ethylidene'-violins with minimum conversion into 'normal'-violins after standing for 15 min in the dark; maximum conversion into 'normal'-violin with minimum degradation required 120 min.

Oxidation with chromic acid (Rüdiger, 1969)

About 50 μg of pigment was dissolved in acetone (0.1 ml), and 1% (w/v) CrO_3 in 1 M- H_2SO_4 (0.2 ml) was added. After standing for 15 h, ethyl acetate (0.2 ml) was added and the mixture shaken. The ethyl acetate extract was removed, evaporated to dryness in a slow stream of N_2 at room temperature (20°C) and the residue taken up in chloroform (0.1 ml). This solution was examined by g.l.c.

Gas-liquid chromatography

The imides obtained by chromic acid oxidation of the bile pigments were chromatographed on a Perkin-Elmer F11 chromatograph by using a glass column (150 cm $\times$ 0.32 cm internal diam.) packed with 4% cyclohexanedimethanol succinate on acid-washed dichlorodimethylsilane-treated Chromosorb W. The carrier gas was N_2 at 60 ml/min. The detector was flame ionization with a hydrogen pressure of 172 kPa and an air pressure of 138 kPa. Authentic samples of methylvinylmaleimide and ethylmethylmaleimide were prepared from protoporphyrin and mesoporphyrin respectively by chromic acid oxidation.

Some experiments using t.l.c. were performed, but were not used for the majority of pigments, because

this involved the use of a spray reagent containing benzidine or *o*-tolidine (Rüdiger, 1969), both of which are carcinogenic.

Preparation of individual pigments

Biliverdin (or glaucobilin) IX α dimethyl ester. Bilirubin (or mesobilirubin) was dehydrogenated with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone in dimethyl sulphoxide, and the product esterified with BF_3 in methanol. The verdin dimethyl ester was purified by passage through a column (10 cm $\times$ 1 cm) of alumina in chloroform. The pigment was eluted in a small volume of chloroform and the solution evaporated to dryness. The residue was crystallized from methanol. The isomeric purity of the product was checked by t.l.c. in solvent systems 1 and 2. Only traces of III α and XIII α pigments were observed.

Biliverdins (or glaucobilins) III α , IX α and XIII α dimethyl ester. Bilirubin (or mesobilirubin) was rearranged by acid catalysis in dimethyl sulphoxide and the crude product was dehydrogenated with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone in dimethyl sulphoxide. The product was esterified with methanolic BF_3 , and the crude product always gave three bands on t.l.c. in solvent system 1 with two developments.

(a) Biliverdins. All bands showed tailing. A small upper yellow-green band, the middle largest green band and a lower blue-green band were biliverdins III α , IX α and XIII α dimethyl ester respectively. The maximum loading for reasonable separation was 10 mg of pigment per 20 cm $\times$ 20 cm t.l.c. plate. Two or three developments were necessary, each band after elution being rechromatographed in the same solvent system and minor components caused by overlap recombined with their respective fractions. Finally, the purity of each isomer was checked by t.l.c. in solvent systems 1, 2 and 3. The compounds were all crystallized from methanol, except biliverdin III α dimethyl ester, which was crystallized from aqueous methanol.

(b) Glaucobilins. The three blue glaucobilin bands did not tail, and loadings up to 20 mg per 20 cm $\times$ 20 cm t.l.c. plate were possible. The upper, middle and lower bands were the III α , IX α and XIII α isomers respectively. Each component was rechromatographed in the same solvent system and checked in solvent systems 1, 2 and 3. The compounds were all crystallized from methanol.

Mesobilirubin IX α dimethyl ester. Glaucobilin IX α dimethyl ester was dissolved in a small volume of chloroform, and about 10 vol. of methanol was added and the pigment reduced with NaBH_4 . After isolation the crude product was purified by passing it through a column (10 cm $\times$ 1 cm) of alumina and eluting the major yellow band with chloroform. Elution was hastened by addition of small increments of methanol

to the chloroform. The crude product was recovered by solvent evaporation and used without further purification for the preparation of dihydrobiliverdins (see below).

Dihydrobiliverdin dimethyl esters of the III α , IX α and XIII α series. Crude mesobilirubin IX α dimethyl ester (from 100 mg of glaucobilin IX α dimethyl ester) and bilirubin (200 mg) were dissolved in dimethyl sulphoxide (60 ml) and coupled by acid catalysis. The products were dehydrogenated by 2,3-dichloro-5,6-dicyano-*p*-benzoquinone and extracted exhaustively into chloroform; this removed most of the free biliverdins, which formed an interfacial precipitate. The chloroform solution, containing glaucobilin dimethyl esters, dihydrobiliverdin monomethyl esters and some free biliverdins, was extracted with dilute aqueous NH₃. The glaucobilin dimethyl esters remained in the chloroform and were separated and set aside for recovery. The aqueous alkaline extract contained the dihydrobiliverdin monomethyl esters and free biliverdins. The solution was acidified and extracted with chloroform. The chloroform solution was washed with water, dried and evaporated. The residue was transferred in chloroform to an alumina column (10 cm $\times$ 1 cm). The eluting solvent was changed to methanol and then gradually to methanolic NH₃ of increasing concentration. The monomethyl esters were slowly eluted, but the free pigments remained at the top of the column. (A less powerful adsorbent might have rendered elution easier, but this was not investigated.) The eluted monomethyl esters were extracted into chloroform after acidification and were then esterified with methanolic BF₃. The product was chromatographed in solvent system 1, with two or three developments. Four major bands were obtained in about equal quantities, the two upper bands being green and the two lower bands being blue-green. Each band was eluted and rechromatographed in solvent system 2. Finally, each product was checked by t.l.c. in solvent systems 1, 2 and 3. All the compounds were crystallized from methanol. The uppermost band was dihydrobiliverdin III α dimethyl ester, the second band was A-dihydrobiliverdin IX α dimethyl ester, the third band was D-dihydrobiliverdin IX α dimethyl ester and the lowest band was dihydrobiliverdin XIII α dimethyl ester. The smallest separation was between the two IX α isomers.

The four dihydrobiliverdin dimethyl esters may also be prepared by dehydrogenation with FeCl₃ of the four corresponding dihydrobiliviolin dimethyl esters, but overall yields were smaller. One advantage, however, is in the preparation of the two IX α isomers uncontaminated by each other, as t.l.c. separation of the two IX α dihydroviolin isomers is easier than that of the corresponding verdins (see below).

*'R*R*-Ethylidene'-dihydroisobiliviolin IX α and 'R*R*-ethylidene'-isomesobiliviolin IX α dimethyl esters.* Bilirubin (100 mg) was reduced rapidly with

sodium amalgam. After removal of excess of amalgam the reaction mixture was added to methanol (200 ml) and dehydrogenated with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone, and then 20% (w/v) anhydrous FeCl₃ in HCl (12 M; 20 ml) was added and the mixture was refluxed for 30 min. The pigments were then extracted into chloroform after addition of water. After water washing, filtration and evaporation of solvent the recovered pigments were esterified with methanolic BF₃.

The pigments were chromatographed in solvent system 1 on five 20 cm $\times$ 20 cm t.l.c. plates with two developments. The upper purple band and the violet band below it were recovered. Small amounts of verdin, and violins of low mobility and urobilins, were observed but not usually recovered. The two main pigments were rechromatographed in solvent system 2 and finally checked for purity in solvent systems 1, 2 and 3. The compounds were crystallized from aqueous acetone. The purple compound is '*R*R*-ethylidene*'-dihydroisobiliviolin IX α dimethyl ester and the violet compound is '*R*R*-ethylidene*'-isomesobiliviolin IX α dimethyl ester. If desired the urobilins may be recovered and recycled to increase the yield.

*'R*R*-Ethylidene'-dihydroisobiliviolin IX α , 'R*R*-ethylidene'-dihydrobiliviolin XIII α , biliviolin III α and biliviolin IX α dimethyl esters (basic violin preparation).* Bilirubin (100 mg) was reduced with sodium amalgam as described above, and after removal of excess of amalgam, bilirubin (100 mg) in 0.1 M-NaOH was added. The total pigments were precipitated by dropwise addition of acetic acid / saturated aqueous sodium acetate (1:4, v/v) in a stream of O₂-free N₂. The precipitate was separated by centrifugation and dissolved in dimethyl sulphoxide (40 ml) and coupled by acid catalysis. The recovered pigment was dehydrogenated with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone in dimethyl sulphoxide and recovered as described for violin contaminated with unwanted verdin. The removal of most of the verdin at this stage was essential to the success of the subsequent t.l.c. separations. The pigments were esterified with methanolic BF₃ and chromatographed in solvent system 1 on ten 20 cm $\times$ 20 cm plates with two developments. The amount of verdin obtained depended mainly on its separation as described above. If there is a large quantity, tailing may obscure the uppermost violin band(s). In addition to variable amounts of violins of low mobility obtained as minor products, there were four main purple bands of high mobility. The first and third bands were a bluer shade of purple than the second and fourth bands. The bands in descending order of mobility were the dimethyl esters of '*R*R*-ethylidene*'-dihydroisobiliviolin IX α , biliviolin III α , '*R*R*-ethylidene*'-dihydrobiliviolin XIII α and biliviolin IX α . The greatest separation was between bands 2 and 3. The descending order of abundance was the dimethyl ester of '*R*R*-ethylidene*'-dihydro-

biliviolin XIII α > 'R*R*-ethylidene'-dihydroisobiliviolin IX α > biliviolin IX α > biliviolin III α . Each band was purified by chromatography in solvent system 2 and checked for purity in solvent systems 1, 2 and 3. The compounds were all crystallized from aqueous acetone.

Variations on the basic preparation described above have yielded all the other violins described. (a) 'R*R*-Ethylidene'-isomesobiliviolin IX α and A-dihydrobiliviolin IX α dimethyl esters: 'R*R*-ethylidene'-mesobiliviolin XIII α and 'endo'-dihydrobiliviolin III α dimethyl esters obtained as a mixture. Reduced bilirubin was coupled with mesobilirubin, and the product dehydrogenated, esterified and chromatographed in solvent system 1. Three major violet bands of high mobility were obtained, with small amounts of verdins and violins of low mobility.

The bands in descending order of mobility were 'R*R*-ethylidene'-isomesobiliviolin IX α , 'R*R*-ethylidene'-mesobiliviolin XIII α and 'endo'-dihydrobiliviolin III α , and A-dihydrobiliviolin IX α , the last being the least abundant and the former two of about equal abundance. All attempts to separate the dimethyl esters of 'R*R*-ethylidene'-mesobiliviolin XIII α and 'endo'-dihydrobiliviolin III α failed. The dimethyl esters of A-dihydrobiliviolin IX α and 'R*R*-ethylidene'-isomesobiliviolin IX α were further purified by t.l.c. in solvent system 2 and checked for purity in solvent systems 1, 2 and 3. The compounds were crystallized from aqueous acetone.

(b) 'Exo'-dihydrobiliviolin III α , A-dihydroisobiliviolin IX α , D-dihydrobiliviolin IX α and 'exo'-dihydrobiliviolin XIII α dimethyl esters. Reduced mesobilirubin was coupled with bilirubin, and the product was dehydrogenated, esterified and chromatographed in solvent system 4 with two developments. Four major purple bands of about equal intensity and low mobility were obtained. The two upper bands were a bluer shade of purple than the lower two bands and the greatest separation was between bands 2 and 3. Each band was further purified in solvent system 2 and finally checked for purity in solvent systems 4, 2 and 3. The compounds were crystallized from aqueous acetone. The bands in order of decreasing mobility were the dimethyl esters of 'exo'-dihydrobiliviolin III α , A-dihydroisobiliviolin IX α , D-dihydrobiliviolin IX α and 'exo'-dihydrobiliviolin XIII α .

(c) Isomesobiliviolin IX α dimethyl ester and mesobiliviolin III α , IX α and XIII α dimethyl esters. Reduced mesobilirubin was coupled with mesobilirubin, and the product was dehydrogenated, esterified and chromatographed in solvent system 2 with two or three developments. Four major violet bands of about equal intensity and low mobility were obtained. In descending order of mobility, these were the dimethyl esters of mesobiliviolin III α , isomesobiliviolin IX α , mesobiliviolin IX α and mesobiliviolin

XIII α . Each compound was chromatographed in solvent system 4, and isomesobiliviolin IX α and mesobiliviolin IX α were then rechromatographed in solvent system 2, as only this system separated these two compounds. Final checks of purity were made in solvent systems 4, 2 and 3. The compounds were crystallized from aqueous acetone.

*'R*R*-Ethylidene'-isomesobiliviolin IX α and 'R*R*-ethylidene'-mesobiliviolin XIII α dimethyl esters.* (a) In the basic violin preparation variation (a), the use of a sodium amalgam of greater than 2% concentration prevented the formation of 'endo'-dihydrobiliviolin III α and A-dihydrobiliviolin IX α dimethyl esters, thus enabling 'R*R*-ethylidene'-mesobiliviolin XIII α dimethyl ester to be obtained free of 'endo'-dihydrobiliviolin III α dimethyl ester.

(b) Bilirubin (100mg) was reduced briefly with 3% sodium amalgam. The product was precipitated by dropwise addition of acetic acid/saturated aqueous sodium acetate (1:4, v/v) and separated by centrifugation at 1000g for 10 min. The residue was dissolved in methanol (10ml), and 20% (w/v) anhydrous FeCl₃ in HCl (12M; 1ml) was added, and the mixture refluxed for 5 min. Methanol (90ml) and 20% (w/v) anhydrous FeCl₃ in HCl (12M; 9ml) were added, and the mixture was refluxed for 30 min. After addition of water the pigments were extracted into chloroform. The chloroform solution was washed with water, filtered and evaporated to dryness. The pigment residue was esterified and the pigment esters were chromatographed in solvent system 1 with two developments. The two major violin bands of high mobility were, in descending order, the dimethyl esters of 'R*R*-ethylidene'-isomesobiliviolin IX α and 'R*R*-ethylidene'-mesobiliviolin XIII α . They were further purified by t.l.c. in solvent system 2 and checked for purity in solvent systems 1, 2 and 3, and crystallized from aqueous acetone.

*'R*S*-Ethylidene'-violins from 'R*R*-ethylidene'-violins.* The 'R*R*-ethylidene'-violin was treated with 0.25M-sodium methoxide. After 15 min the pigments were extracted as described above and chromatographed in solvent system 1. The upper major band was starting material, the middle small band was the corresponding 'R*S*-ethylidene'-violin, and the lowest small band was the corresponding 'normal'-violin. All bands were recovered and the starting material was reprocessed until the quantity of starting material was too low to afford a useful yield of product. The product was further purified by t.l.c. in solvent system 2 and checked for purity in solvent systems 1, 2 and 3. The compounds were crystallized from aqueous acetone. The dimethyl esters of 'R*S*-ethylidene'-dihydroisobiliviolin IX α , 'R*S*-ethylidene'-dihydrobiliviolin XIII α , 'R*S*-ethylidene'-isomesobiliviolin IX α and 'R*S*-ethylidene'-mesobiliviolin XIII α were obtained from those of 'R*R*-ethylidene'-dihydroisobiliviolin IX α , 'R*R*-

ethylidene'-dihydrobiliviolin XIII α , '*R***R*'-ethylidene'-isomesobiliviolin IX α and '*R***R*'-ethylidene'-mesobiliviolin XIII α respectively.

'Normal'-violins from corresponding '*R***R*'-ethylidene'-violins. The dimethyl esters of A-dihydroisobiliviolin IX α , 'exo'-dihydrobiliviolin XIII α , isomesobiliviolin IX α and mesobiliviolin XIII α were prepared from those of '*R***R*'-ethylidene'-dihydroisobiliviolin IX α , '*R***R*'-ethylidene'-dihydrobiliviolin XIII α , '*R***R*'-ethylidene'-isomesobiliviolin IX α and '*R***R*'-ethylidene'-mesobiliviolin XIII α respectively in the same way as the corresponding '*R***S*'-ethylidene'-violins had been prepared. The time of isomerization was 2h. In the preliminary chromatography the upper minor band was starting material, the middle minor band the corresponding '*R***S*'-ethylidene'-compound and the lowest major band was the corresponding 'normal'-violin. The starting material was reprocessed if sufficient was recovered.

Spectroscopy

Electronic spectroscopy. Spectroscopic measurements were made on a Pye-Unicam SP.1800 double-beam recording u.v. spectrophotometer. AnalaR-grade chloroform was used throughout after we had established that Spectrosol-grade chloroform offered no advantage in this work. Pigment was weighed in approx. 100 μ g portions on an electronic micro-balance, the accuracy of which was constantly checked with M.A.R.-grade cyclohexanone 2,4-dinitrophenylhydrazone by using its λ_{max} at 366 nm and extinction coefficient determined by a series of 'macro' weighings. The pigment was dissolved in chloroform in a 5 ml volumetric flask, and spectroscopic measurements were made. In each case a mean of three separate weighings and measurements were obtained.

Mass spectrometry. Mass spectra were obtained on an AEI MS902 mass spectrometer by direct-insertion probe. Better results were obtained if the pigment was put on the probe as a solution in chloroform with evaporation of solvent, rather than as a crystalline solid. The amorphous layer of pigment presumably was better able to vaporize in the heated inlet of the mass spectrometer than was the crystalline form. With some pigments no spectra could be obtained by use of crystalline material, but spectra were obtained in every case by use of an amorphous film.

N.m.r. spectroscopy. The n.m.r. spectra were obtained by pulsed and Fourier-transform n.m.r. spectrometry on a Bruker HFX90 90MHz nuclear-magnetic-resonance spectrometer. The solvent was C²HCl₃, and tetramethylsilane was the internal standard. The quantities of pigment varied from about 0.5mg to 10mg and the number of pulses used for a spectrum was from 256 to about 4000. About one pulse every 2s was used. The spin-decoupling experi-

ments were performed on a Varian HA 100 100 MHz spectrometer.

Discussion of experimental conditions, reactions and procedures

Reduction with sodium amalgam. Brief sodium amalgam reduction with 0.5% sodium amalgam gave products in high yield, some of which were observed in low yield after prolonged reduction with 3% sodium amalgam. This suggested a sequence of events in the sodium amalgam reduction of bilirubin (Scheme 1).

Hydrogenation with NaBH₄. This reagent appears to hydrogenate the central methene bridge in violins and verdins specifically, giving the corresponding rubin. Because rubin esters are easily dehydrogenated by air it is essential, after removal of excess of NaBH₄, to perform the isolation rapidly under N₂.

Dehydrogenation with 2,3-dichloro-5,6-dicyano-p-benzoquinone. This high-redox-potential quinone appeared to dehydrogenate the central methylene bridge in urobilinogens specifically giving urobilins, and in rubins giving verdins. The reaction was carried out in neutral conditions, thus avoiding the acid-catalysed rearrangement that occurred when acidic FeCl₃ was used. In high pigment concentration, however, small amounts of rearrangement products were observed, but these could be minimized by using sufficiently dilute solutions. The addition of acetic acid after dehydrogenation was essential, otherwise decomposition of the product occurred during work-up. The nature and mechanism of this decomposition is not known.

Dehydrogenation with acidic FeCl₃. This reagent slowly dehydrogenated the outer methylene bridges of urobilins and violins, but because of the prolonged elevated temperature and strongly acid conditions some degradation and polymerization occurred; the latter could be minimized by using dilute pigment solutions, since it is an intermolecular reaction, whereas the dehydrogenation depends only on the reagent concentration.

The reagent may also be used for rapid dehydrogenation of the central methylene bridge in urobilinogens and rubins, but because of the strongly acid conditions rearrangement also occurs, particularly in concentrated pigment solution.

Acid-catalysed rearrangement (coupling). This reaction was very fast and occurred most efficiently at high pigment concentration. Prolonged acid treatment caused degradation. The reaction is believed to occur via the formation of a dipyrrolic fragment bearing a carbonium ion (Scheme 2).

Base-catalysed isomerization. '*R***R*'-Ethylidene'- and '*R***S*'-ethylidene'-violins were interconverted and converted into 'normal'-violins at a rate dependent on base concentration and temperature; the

Scheme 1. *Proposed sequence for the reduction of bilirubin by sodium amalgam*
(P = $-\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$)

reactions were independent of pigment concentration. The proposed mechanism is shown in Scheme 3.

The proportion of diastereoisomeric violin was small and was not increased by prolonged base treatment, but the total conversion into 'normal'-violin increased with reaction time. Thus to obtain a good yield of diastereoisomer from '*R***R*'-ethylidene'-violin or '*R***S*'-ethylidene'-violin, the former conversion

only being of practical interest, a short reaction time followed by isolation of the products and reprocessing of starting material is essential to minimize the yield of 'normal'-violin.

Pigment preparations. The pigment preparations described have allowed isolation of pigments individually, or in groups of related compounds for subsequent separation. Methods of preparation of some

Scheme 2. Acid-catalysed rearrangement of $IX\alpha$ tetrapyrrole to symmetrical $III\alpha$ and $XIII\alpha$ products
If a mixture of starting compounds is used, new $IX\alpha$ compounds are among the products.

Scheme 3. Base-catalysed isomerization of ' R^*R^* -ethylidene'- and ' R^*S^* -ethylidene'-violins

IX α pigments involve conditions that do not allow acid-catalysed rearrangement of pigments with a central methylene bridge, thus increasing yields and simplifying isolation. However, for many IX α , and all III α and XIII α , compounds a rearrangement step is necessary.

Certain preparations have been particularly useful for the isolation of pigments in a pure form whose formation by other methods involves tedious and often incomplete separation from other compounds, e.g. formation of 'normal'-IX α isoviolsins by base-catalysed isomerization of the corresponding 'ethylidene'-violsins. The large difference in chromatographic mobility allows easy separation of starting material from product, whereas alternative methods in which 'normal'-IX α violsins are also formed presents a much more difficult chromatographic separation. The preparation by similar methods of the 'R*S*-ethylidene'-violsins is similarly successful, as, although these compounds are formed in reasonable yield after sodium amalgam reduction of bilirubin, their isolation in the presence of other reaction products was very difficult.

The preparation of the four dihydrobiliverdins, although possible by FeCl₃ dehydrogenation of the corresponding dihydroviolsins, has been accomplished in good yield, by making use of the coupling reaction, for the formation of monomethyl esters whose properties are very different from those of either the free acids or the dimethyl esters. This technique ensured that only the desired coupling products would appear in the form of monomethyl esters, whereas all by-products, which would otherwise have been very difficult to separate from the desired products, were in the form of free acids or dimethyl esters. Separation into three groups was thus easily achieved as a first step, followed by separation of the individual members of the desired group as dimethyl esters by t.l.c. (see Scheme 4).

Crystallization. All the verdins crystallized readily from methanol except biliverdin III α dimethyl ester, which crystallized from aqueous methanol. All the violsins crystallized from aqueous acetone, and they were all difficult to crystallize, except 'R*S*-ethylidene'-dihydroisobiliviolin IX α dimethyl ester. Other 'R*R*-ethylidene'- and 'R*S*-ethylidene'-violsins were easier to crystallize than most 'normal'-violsins, of which mesobiliviolin IX α dimethyl ester was the most difficult.

Mass spectrometry. The most stable spectra were those of the verdins (except for that of biliverdin III α dimethyl ester) and the mesoviolsins. The least stable were the 'ethylidene'-dihydroviolsins and biliviolin III α dimethyl ester. The 'normal'-dihydroviolsins were of intermediate stability. Dihydroisobiliviolins with an outer vinyl substituent in the chromophore were always less stable than the corresponding dihydrobiliviolin, with an inner vinyl substituent in the chromophore. The reason for these differences in

stability is not known, but in many cases paralleled the general stability of the compounds.

Structural information has been obtained by study of the n.m.r. spectra of 29 of the 30 compounds, but valuable supporting and complementary information has been obtained by mass- and electronic-spectroscopic studies. Chemical and chromatographic studies have proved valuable in confirming conclusions drawn from the physical data.

Mass spectrometry has proved of complementary value to n.m.r., in providing the molecular weight for all compounds and therefore proving their mass homogeneity. For verdins, little other useful information was obtained, but for violsins, the mass of the major fragment ion, which consists of the chromophore together with the terminal methylene bridge, together with the molecular weight, serves to locate a single vinyl group within or outside the chromophore.

Because of disproportionation of the molecular ion in the violsins and because of more general thermal instability in many of the vinyl compounds, best results were obtained by monitoring the molecular-ion region and the major fragment-ion region in two separate experiments for each compound during the appearance of the mass spectrum. In this way, it was possible to observe the relative intensity and position of the ions as they appeared. Recently field-desorption mass spectra for all these compounds have overcome the problems of disproportionation and confirmed the assigned molecular weights unambiguously.

N.m.r. spectroscopy. High-resolution proton-magnetic-resonance spectra obtained by pulsed and Fourier-transform and, in selected cases, spin-decoupling experiments have defined the relative positions of the protons in the β side chains of the compounds and have provided proof of the nature of several new structural systems. The assignments for the resonances are derived from first principles, from previous successful assignments, and from correlations between spectra of groups of compounds, some of whose structures are known from previous studies.

Electronic spectroscopy. Electronic spectroscopy is of primary value in determining the nature of the chromophore in bile pigments. The wavelength of both the u.v. and the visible maxima of a compound is a direct reflexion of the number of conjugated double bonds in the chromophore. Thus violsins are readily distinguished from verdins by the much shorter wavelength of their absorption maxima. Replacement of ethyl substituents by vinyl groups only produces a marked effect in the spectrum if the vinyl double bond is conjugated with the chromophore, but a clear difference is observed between linearly conjugated and cross-conjugated vinyl substituents, which is of value in classification.

Catalytic hydrogenation. Catalytic hydrogenation in formic acid specifically converts vinyl groups into

Scheme 4. Preparation of the four dihydrobiliverdin dimethyl esters of the III α , IX α and XIII α series from bilirubin IX α , and mesobilirubin IX α dimethyl ester

Abbreviation: Cl₂(CN)₂BzQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone.

ethyl groups in both violins and verdins. In this way all vinyl compounds could be converted into their ethyl ('meso') analogues and the latter identified by comparison with authentic compounds by t.l.c. This method further confirmed the relationships implicit in the physical findings.

FeCl₃ dehydrogenation. This technique has been used to relate violins to their corresponding verdins by conversion of the former into the latter and identification of the verdin product by t.l.c. by using known verdins as standards.

Chromatography. T.l.c. is primarily used to isolate the compounds in pure form, but structural information can also be gained from the relationship between *R_F* values and various chemical features in the groups of compounds.

Results and Discussion

By using the new and improved preparative techniques, three violins and six verdins previously described by Stoll & Gray (1970) have again been prepared and precise physicochemical studies have been performed to provide reference data to aid in the interpretation of the results on the four new verdins and 17 new violins. In all the work described below the synthetic mesobiliviolin and isomesobiliviolin IX α dimethyl esters were shown to be identical with compounds 21 and 20 respectively.

Mass spectrometry

Table 2 shows the results of mass-spectral analysis of all the compounds. Because many of the spectra

Table 2. *Mass-spectral data for the pigment dimethyl esters*

The data were obtained by monitoring the molecular-ion and major fragment-ion region in two separate experiments for each compound.

Compound no.	Molecular ion		Major fragment ion		
	Mass	No. of vinyl groups	Mass	Consisting of the chromophore and ...	No. of vinyl groups
1	610	2	—	—	—
2	610	2	—	—	—
3	610	2	—	—	—
4	612	1	—	—	—
5	612	1	—	—	—
6	612	1	—	—	—
7	612	1	—	—	—
8	614	0	—	—	—
9	614	0	—	—	—
10	614	0	—	—	—
11		Not investigated			
12	612	2	490	CH ₂	1
13	614	1	490	CH ₂	1
14		Not investigated			
15	614	1	490	CH ₂	1
16	614	1	492	CH ₂	0
17	614	1	490	CH ₂	1
18	614	1	490	CH ₂	1
19	616	0	492	CH ₂	0
20	616	0	492	CH ₂	0
21	616	0	492	CH ₂	0
22	616	0	492	CH ₂	0
23	614	1	491	CH ₃	1
24	614	1	491	CH ₃	1
25	614	1	491	CH ₃	1
26	614	1	491	CH ₃	1
27	616	0	493	CH ₃	0
28	616	0	493	CH ₃	0
29	616	0	493	CH ₃	0
30	616	0	493	CH ₃	0

were obtained by monitoring the molecular-ion region and, for violins, the principal fragment-ion region, in two separate experiments for each compound, only the molecular ion for verdins and the molecular ion and principal fragment ion for violins is shown. The verdin spectra are compatible with compounds 1, 2 and 3 being biliverdin dimethyl esters, compounds 4, 5, 6 and 7 being dihydrobiliverdin dimethyl esters and compounds 8, 9 and 10 being mesobiliverdin (glucobilin) dimethyl esters. All violins showed the disproportionation previously described (Stoll & Gray, 1970).

The violin spectra were compatible with the following dimethyl esters of bile pigments: compound 12, a biliviolin; compounds 19–22 and 27–30, mesobiliviolins; compounds 13, 15, 17, 18 and 23–26, dihydrobiliviolins with extra unsaturation in the chromophore; compound 16, a dihydrobilirubin with extra unsaturation outside the chromophore; compounds 11 and 14 were not investigated.

Recently the field-desorption mass spectra of all these compounds have been obtained, and they confirm in every case the molecular weights for the compounds concluded from the electron-impact spectra (Cooper *et al.*, 1975).

N.m.r. spectrometry

The ¹H n.m.r. spectra are shown in Figs. 1–7 and 9; detailed data are given in Tables 3, 4 and 5. In the following text all bile pigments discussed are as dimethyl esters, and all signals are expressed as δ (p.p.m.).

Biliverdins. The n.m.r. spectra of the dimethyl esters of biliverdins III α , IX α and XIII α have been described previously (Bonnett & McDonagh, 1970). However, their spectra (Fig. 1), obtained by pulsed and Fourier-transform n.m.r., are included here, since the quality of these spectra makes more precise interpretation possible, and they provide a useful

Fig. 1. Fourier-transform n.m.r. spectra at 90 MHz in C^2HCl_3 of biliverdins, (a) biliverdin III α dimethyl ester, (b) biliverdin IX α dimethyl ester and (c) biliverdin XIII α dimethyl ester

Each proton, or group of equivalent protons, in the molecule is numbered in the structures shown. N.m.r. signals arising from these protons are indicated in the spectrum by the appropriate proton number. The total width of multiplet signals is indicated by horizontal brackets. Me_4Si tetramethylsilane.

basis for interpretation of the spectra of the new vinyl compounds.

Compounds 1 and 3 show relatively simple spectra, readily interpreted together as those for biliverdins III α and XIII α respectively. One main difference lies in the positions of the singlet signals from the ending β -methyl substituents. In the III α compound 1 this signal is downfield (2.21) relative to that (1.90) in the XIII α compound 3, as expected because of the greater deshielding effect of the chromophore on substituents in inner positions of the molecule. The other main differences in the spectra are the signals of the vinyl substituents. An interpretation of these differences has been described previously for the n.m.r. spectra of vinyl and isovinyl azobilirubin methyl esters (Jansen & Stoll, 1971). The vinyl signals in the spectrum of compound 1 with three distinct double doublets (6.58, 5.52, 6.08) are characteristic for an outer vinyl substituent (III α structure), and those in the spectrum of compound 3, in which an isolated low-field double doublet (6.68) and two characteristic high-field doublets (5.67, 5.69) are characteristic for an inner vinyl substituent (XIII α structure). On the basis of these two spectra the spectrum of biliverdin IX α (compound 2) can readily be interpreted as shown in Table 3. The assignments for the four methyl signals were confirmed by a lanthanide shift experiment (M. S. Stoll, unpublished work).

Two minor effects of interest in these spectra are (a) the anomalously high field position (6.08) of the double doublet from the *trans*-geminal proton of the vinyl groups in the spectrum of biliverdin III α when compared with the position in the spectra of other vinyl verdins, which is probably due to steric interaction of the two vinyl groups, providing indirect evidence for a planar U-shaped molecule in the verdins, and (b) the broadening of the signals from the outer methyl-group protons (1.90, 1.85) and the outer bridge protons 5 and 15 in biliverdin XIII α and of proton 5 in the IX α isomer. This is probably due to a long-range coupling of low magnitude. A similar effect is seen in all bile-pigment spectra.

Mesobiliverdins. The n.m.r. spectra of compounds 8, 9 and 10, mesobiliverdins (glucobilins) III α , IX α and XIII α respectively have been described previously (Stoll & Gray, 1970), but pulsed and Fourier-transform spectrometry have improved the quality of the spectra. The interpretation of the spectra is as shown in Fig. 2 and Table 3; the main additional features shown, which were not previously interpretable because of poor resolution and signal-to-noise ratio, are the quartets from the methylene protons 2¹ and 18¹ of the β -ethyl substituents and the separate signals from the bridge protons 5 and 15 in the spectrum of mesobiliverdin IX α . Again there is a broadening of the signals from the outer bridge protons 5 and 15 in glucobilin XIII α and 5 in the IX α isomer, and also

of those from the outer β -methyl protons 2¹ and/or 18¹ in both isomers [signals at 5.90, 1.80 (IX α) and 5.94, 1.82 (XIII α)].

Dihydrobiliverdins. The n.m.r. spectra of compounds 4, 5, 6 and 7 (Table 3 and Fig. 3) are readily interpreted by using the spectra of compounds 1, 2, 3 and 8, 9 and 10 as models. The n.m.r. spectra show many features in common, but differ in (a) the type of vinyl signals, (b) the position of two of the four methyl signals and (c) the position of the signals from the single ethyl group.

Compounds 4 and 5 each contain similar vinyl signals characteristic of an outer vinyl substituent [cf. spectra of compounds 1 and 2, signals at 6.53, 5.41, 6.16 (compound 4) and 6.55, 5.44, 6.11 (compound 5) and a similar low-field methyl signal at 2.18 and 2.20 (compounds 4 and 5 respectively)] from the inner methyl group in the end ring bearing a vinyl substituent. Compounds 6 and 7 gave similar vinyl signals characteristic of an inner vinyl substituent (cf. spectra of compounds 2 and 3), signals at 6.64, 5.64, 5.66 (compound 6) and 6.64, 5.64, 5.67 (compound 7) and a similar high-field methyl signal at 1.93 (compounds 6 and 7) from the outer methyl group in the end ring bearing a vinyl substituent. Compounds 4 and 6 each give a similar low-field methyl signal at 2.09 and 2.10 respectively and similar high-field ethyl signals at 2.24 and 1.05 (compound 4) and 2.28 and 1.07 (compound 6) from an end ring containing an inner methyl substituent and outer ethyl substituent. Compounds 5 and 7 each contain a similar high-field methyl signal at 1.78 and 1.81 respectively and a similar low-field ethyl signal at 2.54 and 1.22 (compound 5) and 2.51 and 1.22 (compound 7) from an end ring containing an outer methyl and an inner ethyl substituent. Such findings could be produced either by dihydrobiliverdins or by mixtures of mesobiliverdins and biliverdins. However, the chromatographic properties and mass and electronic spectra are compatible only with the compounds being dihydrobiliverdins; thus compound 4 has in one end ring an outer vinyl and an inner methyl substituent and in the other end ring an outer ethyl and an inner methyl group and thus is dihydrobiliverdin III α . Compound 5 has in its outer rings outer vinyl and methyl substituents and inner methyl and ethyl substituents and is thus A-dihydrobiliverdin IX α . Compound 6 has inner vinyl and methyl substituents and outer methyl and ethyl substituents and is therefore D-dihydrobiliverdin IX α , and compound 7 has inner vinyl and ethyl substituents and two outer methyl substituents and thus is dihydrobiliverdin XIII α .

Mesobiliviolins. The n.m.r. spectra of compounds 21 and 22 (mesobiliviolins IX α and XIII α) have been described previously (Stoll & Gray, 1970), but are presented here with those of compound 19 (mesobiliviolin III α) and compound 20 (isomesobiliviolin IX α) for complete interpretation (Fig. 4 and Table 4).

3	6.68 (11)17 ¹ ,17 ^{2a} (9)17 ¹ ,17 ^{2b} d.d.	5.77 (<1)17 ^{2a} ,17 ^{2b} (13)17 ¹ ,17 ^{2a} d.d.	5.69 (<1)17 ^{2a} ,17 ^{2b} (17)17 ¹ ,17 ^{2b} d.d.	1.90	6.53 (10)18 ¹ ,18 ^{2a} (19)18 ¹ ,18 ^{2b} d.d.	5.41 (4)18 ^{2a} ,18 ^{2b} (11)18 ¹ ,18 ^{2a} d.d.	6.16 (4)18 ^{2a} ,18 ^{2b} (17)18 ¹ ,18 ^{2b} d.d.	6.04	6.80	6.04
4	2.18							5.91	6.77	5.99
5	2.20				6.55 (12)18 ¹ ,18 ^{2a} (19)18 ¹ ,18 ^{2b} d.d.	5.44 (3)18 ^{2a} ,18 ^{2b} (11)18 ¹ ,18 ^{2a} d.d.	6.11 (4)18 ^{2a} ,18 ^{2b} (21)18 ¹ ,18 ^{2b} d.d.	6.05	6.83	5.99
6	2.10				2.28 (8)18 ¹ ,18 ² q.	1.07 (8)18 ¹ ,18 ² t.		6.01	6.72	5.86
7	2.51 (7)17 ¹ ,17 ² q.	1.22 (8)17 ¹ ,17 ² t.			1.81			6.05	6.75	5.91
8	2.09				2.27 (7)18 ¹ ,18 ² q.	1.06 (7)18 ¹ ,18 ² t.		5.88	6.74	5.88
9	2.07				2.26 (7)18 ¹ ,18 ² q.	1.06 (8)18 ¹ ,18 ² t.		5.90	6.74	5.87
10	2.52 (7)17 ¹ ,17 ² q.	1.21 (7)17 ¹ ,17 ² t.			1.82			5.94	6.77	5.94

The spectra are readily interpreted by reference to the spectra of mesobiliverdins III α , IX α and XIII α (compounds 8, 9 and 10) and to the spin-decoupling experiments described below on compound 27, which is an 'R*R*-ethylidene'-mesobiliviolin of closely related structure. The spectra of compounds 19, 20, 21 and 22 all show signals within 0.01 of 3.66, 3.64, 2.91, 2.51, 2.07 and 1.99 from the β -substituents of the centre two rings and at about 3.17 and 2.85 from the methylene-bridge protons, but differ in the positions of two of the four methyl signals and the two ethyl signals. There are also significant differences in the position of the signal from the NH proton of the unconjugated end ring. Compounds 19 and 20 each show similar high-field ethyl signals at 2.39 and 1.12 (compound 19) and 2.40 and 1.13 (compound 20) and low-field methyl signals at 2.10 (compounds 19 and 20), but differ in that the second set (presumably position 2 or 3) of ethyl signals is at high field (2.13 and 0.82) in compound 19 and at low field (2.32 and 1.07) in compound 20 and the second, presumably position 2 or 3, methyl signal, is at low field (1.88) in compound 19 and at high field (1.70) in compound 20. Compounds 21 and 22 each give similar low-field ethyl signals at 2.54 and 1.19 and high-field methyl signals at 1.95 (compound 21) and 1.94 (compound 22), but differ in that the second set of ethyl signals is at high field (2.12 and 0.82) in compound 21 and at low field (2.32 and 1.06) in compound 22 and the second methyl signal is at low field (1.88) in compound 21 and at high field (1.69) in compound 22. These second ethyl and methyl signals in compounds 21 and 22 have similar chemical shifts to the second ethyl and methyl signals in compounds 19 and 20 respectively. The interpretation of high- and low-field signals is the same as in the mesobiliverdins, i.e. outer substituents give signals at higher field than do corresponding inner substituents. In addition, in violins, signals in the unconjugated ring give, as expected, signals at higher field than those attached to the deshielding π -bonded chromophore. In the description above the second set of signals is, taken together, at higher field than the first set and thus confirms the assignment of the second set of methyl and ethyl signals to the 2 and 3, or 17 and 18, substituents on the unconjugated end ring. Thus compound 19 contains two outer ethyl and two inner methyl substituents in the end rings, and is therefore mesobiliviolin III α . Compound 20 contains an outer ethyl and an inner methyl substituent in the outer ring of the chromophore, and an outer methyl and an inner ethyl substituent in the unconjugated end ring, and is therefore isomesobiliviolin IX α . Compound 21 contains an outer methyl and an inner ethyl group in the end ring of the chromophore and an outer ethyl and an inner methyl group in the unconjugated end ring and is thus mesobiliviolin IX α . Compound 22 contains two outer methyl and two inner ethyl substi-

12	2.06	6.57 (10)181,182a (20)181,182b d.d.	5.40 (4)182a,182b (10)181,182a d.d.	~6.23 (4)182a,182b (-)-181,182b d.d.	6.19	6.92 3.24 (-) (-) (5)15,16 (16,-)gem. 2d.d.	4.40 m. (broad)	7.33
13*	2.19	6.63 (12)181,182a (20)181,182b d.d.	5.49 (3)182a,182b (10)181,182a d.d.	6.30 (3)182a,182b (17)181,182b d.d.	4.32 m. (broad) 3.18 (3.14) 2.91 (2.95) (4)4,5 (17,25)gem. 2d.d.	6.80 6.04		7.37
14					Not investigated			
15*	2.19	6.61 (11)181,182a (17)181,182b d.d.	5.49 (4)182a,182b (11)181,182a d.d.	6.30 (4)182a,182b (18)181,182b d.d.	4.40 m. (broad) 3.19 (3.10) 2.83 (2.92) (3.14,5) (15,28)gem. 2d.d. 5.95	6.80 6.01		7.16
16	2.03	6.42 (10)181,182a (19)181,182b d.d.	5.32 (4)182a,182b (10)181,182a d.d.	6.13 (4)182a,182b (17)181,182b d.d.		6.78 3.19 (-) (-) (5)15,16 (16,-)gem. 2d.d.	4.36 (5)15,16 t. (broad)	7.20
17	1.87	~2.12 (7)181,182 q.	0.81 (8)181,182 t.		6.08	6.77 3.17 (-) (-) (5)15,16 (16,-)gem. 2d.d.	4.28 (5)15,16 m. (broad)	7.46
18	2.32 (7)171,172a q.	1.69			6.08	6.79 3.19 (3.11) 5.86 (2.92) (5)15,16 (13,30)gem. 2d.d.	4.38 (5)15,16 t. (broad)	7.25
19*	2.10	2.39 (8)181,182 q.	1.12 (7)181,182 t.		3.16 (3.10) 2.87 (2.93) (4)4,5 (16,27)gem. 2d.d.	6.78 5.96		7.40
20*	2.10	2.40 (8)181,182 q.	1.13 (8)181,182 t.		4.41 (7)4,5 t. (broad) 3.18 (3.09) 2.83 (2.91) (5)4,5 (16,31)gem. 2d.d.	6.79 5.93		7.22
21	1.88	~2.12 (8)181,182 q.	0.82 (7)181,182 t.		5.98	6.79 3.17 (3.13) 2.91 (2.95) (5)15,16 (16,24)gem. 2d.d.	4.32 (5)15,16 t. (broad)	7.56
22*	2.54 (7)171,172 q.	1.94			3.16 (3.09) 2.85 (2.92) (5)4,5 (15,28)gem. 2d.d.	6.79 5.97		7.31

Table 5. *N.m.r. data at 90 MHz in C²HCl₃ for dihydrotolins and mesobilitolins containing ethylidene groups*
 For details of layout and abbreviations, see the legends to Tables 3 and 4.

Compound no.	End ring										Dipyrromethene									
	2		2a		2b		3		7		8		12		13					
23*	2	2 ¹	1.05				31	3 ^a	3 ^b	71	81	82	84	121	122	124				
		(7)2,2 ¹	d.				(7)31,32	(7)31,32		1.89	2.89	2.55	3.64	(6)121,122	(5)121,122	3.68				
							q. (broad)	d.d.		(6)81,82	(5)81,82	t.	t.	t.						
24	2.04						6.64	(12)31,32a	(13)31,32b	2.07	2.89	2.55	3.67	(7)121,122	(6)121,122	3.62				
							d.d.	d.d.	d.d.	(7)81,82	(6)81,82	t.	t.	t.						
25*	0.64	(8)2,2 ¹	d.				~5.40	(-)-31,32	(6)31,32	1.92	2.91	2.56	3.64	(7)121,122	(6)121,122	3.67				
							q. (broad)	d. (broad)	d. (broad)	(17)81,82	(6)81,82	t.	t.	t.						
26	2.07						6.64	(12)31,32a	(13)31,32b	2.07	2.91	2.54	3.66	(8)121,122	(6)121,122	3.64				
							d.d.	d.d.	d.d.	(8)81,82	(6)81,82	t.	t.	t.						
27*	2.10	1.06	m.				5.30	(7)31,32	(4)2,32	1.89	2.88	2.55	3.63	(7)121,122	(6)121,122	3.67				
		(7)2,2 ¹	d.				q. (broad)	d.d.	d.d.	(7)81,82	(6)81,82	t.	t.	t.						
28	1.95						2.51	(6)31,32	(8)31,32	2.05	2.89	2.55	3.64	(7)121,122	(5)121,122	3.62				
							q.	t.	t.	(7)81,82	(5)81,82	t.	t.	t.						
29*	0.64	(8)2,2 ¹	d.				5.41	(7)3,31	(7)3,31	1.91	2.91	2.55	3.64	(8)121,122	(6)121,122	3.66				
							q. (broad)	d. (broad)	d. (broad)	(8)81,82	(6)81,82	t.	t.	t.						
30	1.94						2.51	(6)31,32	(7)31,32	2.07	2.90	2.55	3.66	(7)121,122	(6)121,122	3.64				
							q.	t.	t.	(7)81,82	(6)81,82	t.	t.	t.						
Compound no.	End ring										Bridge atoms									
23*	17		17a		17b		18		18a		4		5		10		15		16	
	171	2.19	172a		172b		18	181	182a	182b	4.66	m. (broad)	3.14	(3.07)	6.75	6.03			N	
									5.47	6.28			(2.91)	(2.84)					8.51	
									(4)182a,182b	(3)182a,182b			(4)4,5	(15,27)gem.						
									d.d.	d.d.			2d.d.	2d.d.						
24	5.32	(7)171,172	1.74				1.05	181	182a	182b	6.76		6.09	3.13	4.64					
		q. (broad)	(7)171,172				d.							(3.08)	m. (broad)					
														2.87						
														(2.92)						
														(4)15,16						
														(14,23)gem.						
														2d.d.						

25*	2.20	6.61 (10)18 ¹ ,18 ^{2a} (17)18 ¹ ,18 ^{2b} d.d.	5.49 (3)18 ^{2a} ,18 ^{2b} (10)18 ¹ ,18 ^{2a} d.d.	6.29 (4)18 ^{2a} ,18 ^{2b} (18)18 ¹ ,18 ^{2b} d.d.	4.74 m. (broad)	3.17 (3.12) 2.93 (2.97) (4)4.5 (14,23)gem. 2d.d.	6.80	6.02	8.06	
26	5.41 (6)17 ¹ ,17 ² q. (broad)	1.73 (7)17 ¹ ,17 ² (<1)17 ² ,18 d. (broad)	0.64 (8)18,18 ¹ d.				6.81	3.16 (~3.11) ~2.92 (~2.96) (4)15,16 (~,-)gem. 2d.d.	4.72 m. (broad)	8.12
27*	2.10	2.39 (8)18 ¹ ,18 ² q.	1.13 (7)18 ¹ ,18 ² t.		4.64 m. (broad)	3.11 (3.06) 2.85 (2.90) (4)4.5 (14,24)gem. 2d.d.	6.73	5.93	8.54	
28	5.32 (8)17 ¹ ,17 ² q. (broad)	1.05 (8)18,18 ¹ d.				5.95	6.73	3.14 (-) (-) (-) (4)15,16 (14,-)gem. 2d.d.	4.62 m. (broad)	8.58
29*	2.10	2.40 (8)18 ¹ ,18 ² q.	1.13 (8)18 ¹ ,18 ² t.		4.72 m. (broad)	3.16 (3.11) 2.90 (2.95) (4)4.5 (14,23)gem. 2d.d.	6.78	5.94	8.09	
30	5.41 (7)17 ¹ ,17 ² q. (broad)	1.73 (6)17 ¹ ,17 ² (<1)17 ² ,18 d. (broad)	0.62 (8)18,18 ¹ d.			5.96	6.79	3.17 (3.11) 2.90 (2.95) (4)15,16 (14,24)gem. 2d.d.	4.72 m. (broad)	8.21

Fig. 2. Fourier-transform n.m.r. spectra at 90 MHz in C^2HCl_3 of mesobiliverdins, (a) mesobiliverdin III α dimethyl ester, (b) mesobiliverdin IX α dimethyl ester and (c) mesobiliverdin XIII α dimethyl ester. Me_4Si , tetramethylsilane. Each proton, or group of equivalent protons, in the molecule is numbered in the structures shown. N.m.r. signals arising from these protons are indicated in the spectrum by the appropriate proton number. The total width of multiplet signals is indicated by horizontal brackets.

tuent in the end rings and is thus mesobiliviolin XIII α .

The positions of the signals from the NH protons of the conjugated end ring substantiate the above assignments. Thus upfield shifts of similar magnitude are observed in comparing the spectra of compounds 19 and 20 (7.40 to 7.22) and those of compounds 21 and 22 (7.56 to 7.31), in both cases due to similar changes in the β position of the unconjugated end ring. Smaller downfield shifts of similar magnitude are observed in comparing the spectra of compounds 19 and 21 (7.40 to 7.56) and those of compounds 20 and 22 (7.22 to 7.31) owing to similar changes in the β positions in the chromophore, which, being more remote from the NH proton in the unconjugated end ring, produces a smaller effect. Other points of interest in the spectra also supporting the assignments are as follows.

(a) A small downfield shift of the outer methene-bridge proton signal in the spectra of compounds 21 and 22 (5.98 and 5.97 respectively) compared with those of compounds 19 and 20 (5.96 and 5.93 respectively), in agreement with a change in the substituent nearest this proton, e.g. at the inner β position of the end ring of the chromophore.

(b) A small downfield shift of the α -proton signal of the unconjugated end ring in the spectra of compounds 20 and 22 (4.41 and 4.39 respectively), compared with those of compounds 19 and 21 (4.31 and 4.32 respectively), in agreement with a change in the substituent nearest this position, i.e. the inner β position of the unconjugated end ring.

(c) There is marked broadening of the high-field methyl signal in the spectra of compounds 20 and 22 (1.70 and 1.69 respectively), undoubtedly due to coupling to the α -proton of the unconjugated end ring, thus supporting the assignment of these methyl signals to an outer methyl substituent in the unconjugated end ring in these compounds.

Dihydrobiliviolins with a vinyl group conjugated to the chromophore. Compounds 13, 15, 17 and 18 were shown by mass spectrometry to be dihydrobiliviolins containing the extra double bond in relation to mesobiliviolins, in the chromophore. The n.m.r. spectra (Fig. 5 and Table 4) of the four compounds are easily interpreted by reference to the spectra of compounds 4, 5, 6, 7 (dihydrobiliverdins), 8, 9, 10 (mesobiliverdins), and 19, 20, 21 and 22 (mesobiliviolins).

If compound 13 is 'exo'-dihydrobiliviolin III α , it bears the same relationship to mesobiliviolin III α (compound 19) as dihydrobiliverdin III α (compound 4) bears to mesobiliverdin III α (compound 8). Compound 13 also bears the same relationship to compound 4 as compound 19 does to compound 8. Compared with the spectrum of compound 19, that of compound 13 contains no low-field signal corresponding to a chromophore ethyl group, but contains signals characteristic of a vinyl group in the outer

position of an end ring at 6.63, 5.49 and 6.30. There is also a small downfield shift for the methyl signal at 2.19 (compound 13) compared with 2.10 (compound 19), because the methyl group in compound 13 is on the same ring as the deshielding vinyl group. Similarly the signal at 6.04 of the outer methene-bridge proton (position 15) of compound 13 is downfield from the corresponding signal at 5.96 of compound 19, but there is no comparable shift for the signals of the methylene-bridge proton (position 5) at 3.18 and 2.91 (compound 13) compared with those at 3.16 and 2.87 (compound 19). The vinyl group must therefore be in the end ring of the chromophore rather than the unconjugated end ring. Compared with the spectrum of compound 8, that of compound 4 contains only one ethyl signal, but has in addition a vinyl signal characteristic of a vinyl group in the outer position of an end ring (signals at 6.53, 5.41, 6.16). There are also small downfield shifts for one methyl signal at 2.18 (compound 4) compared with 2.09 (compound 8), and for one outer bridge proton (position 15) at 5.99 (compound 4) compared with 5.88 (compound 8). Some more subtle but significant effects emerge from a comparison of the spectra of compounds 13 and 4 and those of compounds 19 and 8. Comparing the spectrum of compound 4 with that of compound 13, there is a large upfield shift of the ethyl signals (2.24 and 1.05 to 2.11 and 0.82) and of one methyl signal (2.09 to 1.99), and a smaller upfield shift of the signal of one of the three other methyl groups (2.09 to 2.00) due to the decreased conjugation. There is, however, a small downfield shift of the two remaining bridge protons of the chromophore (5.91 to 6.04 and 6.77 to 6.80) and of the vinyl signals, the proton signals of which are not all shifted to the same extent, 6.63 (α), 5.49 (*cis* β) and 6.30 (*trans* β) (compound 13), compared with 6.53 (α), 5.41 (*cis* β) and 6.16 (*trans* β) (compound 4). The explanation of this is not clear, but a parallel effect is seen in comparing the spectrum of compound 8 with that of compound 19, in which there is also a large upfield shift of one ethyl (2.27 and 1.06 to 2.13 and 0.82) and one methyl signal (2.09 to 1.88); a smaller upfield shift of one of the three other methyl signals (2.07 to 1.99), but a small downfield shift of the two remaining bridge-proton signals (5.88 to 5.96 and 6.74 to 6.78) and of the remaining ethyl signals with non-identical shifts of the methyl 1.06 (compound 8) to 1.12 (compound 19) and methylene (2.27 to 2.39) proton signals.

Thus the relationships between compounds 4, 8, 13 and 19 are very clear. The identity of compound 13 is confirmed. Comparison of the spectra of compounds 13, 15, 17 and 18 shows that there is a similar set of relationships to those existing between compounds 4, 5, 6 and 7 and between compounds 19, 20, 21 and 22. Thus the spectra of compounds 13 and 15 each contain vinyl signals characteristic of an outer vinyl substituent at 6.63, 5.49 and 6.30 (compound 13) and

Fig. 3. Fourier-transform n.m.r. spectra at 90 MHz in C^2HCl_3 of dihydrobiliverdins, (a) dihydrobiliverdin III α dimethyl ester (b) A-dihydrobiliverdin IX α dimethyl ester, (c) D-dihydrobiliverdin IX α dimethyl ester and (d) dihydrobiliverdin XIII α dimethyl ester

Me₄Si, tetramethylsilane. Each proton, or group of equivalent protons, in the molecule is numbered in the structures shown. N.m.r. signals arising from these protons are indicated in the spectrum by the appropriate proton number. The total width of multiplet signals is indicated by horizontal brackets.

6.61, 5.49 and 6.30 (compound 15) and a low-field methyl signal 2.19 (compounds 13 and 15) characteristic of an inner end-ring group at position 17 of a violin chromophore. They differ in that the spectrum of compound 15 contains a low-field signal at 2.32 and 1.07 and a high-field signal at 1.70 from inner ethyl and outer methyl groups at positions 3 and 2 respectively on an unconjugated end ring; in contrast, compound 13 gives high-field signals at 2.11 and 0.82 and a low-field signal at 1.89 from outer ethyl and inner methyl groups respectively at positions 3 and 2 on an unconjugated end ring. Similarly, the spectra of compounds 17 and 18 each contain vinyl signals at 6.63, 5.64 and 5.68 (compound 17) and at 6.64, 5.65 and 5.69 (compound 18), characteristic of an inner vinyl group attached to an end ring, and a high-field methyl signal at 2.05 (compound 17), 2.07 (compound 18), corresponding to a group at position 2 of a violin chromophore, but differ in that the spectrum of compound 17 contains a high-field ethyl signal at 2.12 and 0.81 and a low-field methyl signal at 1.87 similar to those in compound 13, and compound 18 contains a low-field ethyl signal at 2.32 and 1.07 and a high-field methyl signal at 1.69 similar to those in compound 15. Owing to deshielding by the neighbouring vinyl groups, compounds 17 and 18 each exhibit a small downfield shift of the signal at 6.08 due to the outer methene-bridge proton at position 5 compared with that in compounds 13 and 15 at position 15.

The relationship between the spectra of compounds 5, 9, 15 and 20, between those of compounds 6, 9, 17 and 21 and between those of compounds 7, 10, 18 and 22 respectively are similar to those between the spectra of compounds 4, 8, 13 and 19 respectively described above, and thus the same structural relationships exist within the four sets of four compounds. The same unexplained downfield shift of signals from the outer group of the chromophore in comparing verdin with violin is observed in all cases. This must relate to a steric difference between verdins and violins, probably in the interactions of the ends of the molecule. Compound 13 is thus 'exo'-dihydrobiliviolin III α , compound 15 is A-dihydroisobiliviolin IX α , compound 17 is D-dihydrobiliviolin IX α , and compound 18 is 'exo'-dihydrobiliviolin XIII α .

Violins with a vinyl group in the unconjugated end ring. The mass spectrum of compound 12 showed that it was a biliviolin containing two extra double bonds in relation to mesobiliviolins, one within and one outside the chromophore. The mass spectrum of compound 16 shows that it is a dihydrobiliviolin containing the one extra double bond outside the chromophore. The n.m.r. spectra of compounds 11, 12 and 16 (Fig. 6 and Table 4) contain similar signals at 6.48, 5.38 and 6.18 (compound 11), 6.52, 5.40 and 6.23 (compound 12) and 6.42, 5.32 and 6.13 (compound 16) characteristic of an end-ring outer vinyl substituent, but at higher field than those in any of the compounds described so far. This is clearly ex-

Fig. 4. Fourier-transform n.m.r. spectra at 90 MHz in C^2HCl_3 of mesobiliviolins, (a) mesobiliviolin III α dimethyl ester, (b) isomesobiliviolin IX α dimethyl ester, (c) mesobiliviolin IX α dimethyl ester and (d) mesobiliviolin XIII α dimethyl ester Me_4Si , tetramethylsilane. Each proton, or group of equivalent protons, in the molecule is numbered in the structures shown. N.m.r. signals arising from these protons are indicated in the spectrum by the appropriate proton number. The total width of multiplet signals is indicated by horizontal brackets.

plained if the vinyl group is in the outer position of the unconjugated end ring of a violin. The remaining features of the spectra are explained by comparing those of compounds 11 and 13, those of compounds 12 and 17 and those of compounds 16 and 21.

Comparing the spectra of compounds 11 and 13, the former has every signal in common with the latter, except those that can be assigned to the substituents of the unconjugated end ring, i.e. all signals from chromophore substituents are similar. The spectra therefore differ in the absence of an ethyl signal, the presence of the high-field outer vinyl signal described above and the downfield shift due to deshielding by the neighbouring vinyl group of the signal from the inner methyl substituent from 1.89 (compound 13) to 2.07 (compound 11) of the unconjugated end ring.

The existence of compound 14 as a mixture with compound 28, from preparations in which both compounds were formed, was determined from n.m.r. spectroscopy. The characteristic signals from an outer vinyl group in an unconjugated end ring, described above, were superimposed on the spectrum of compound 28. Additional evidence came from treatment of compounds 14 and 28 with sodium methoxide, which gave, apart from the products of isomerization of compound 28, large quantities of verdin, as expected from compound 14 (see under 'Base-catalysed isomerization').

Similar relationships are observed between the spectra of compounds 12 and 17 and between those of

compounds 16 and 21. The signals from chromophore substituents are similar for compounds 12 and 17 and for compounds 16 and 21. In addition, the positions of the unconjugated end-ring substituents are similar in compounds 13, 17 and 21, which have each been shown to contain outer ethyl and inner methyl substituents, and similar in compounds 11, 12 and 16, which therefore each contain outer vinyl and inner methyl substituents. Compound 11 is therefore biliviolin III α , compound 12 is biliviolin IX α and compound 16 is A-dihydrobiliviolin IX α .

Similar relationships can be observed in the spectra of the three sets of four compounds: 11, 1, 13, 4; 12, 2, 17, 6; and 16, 5, 21, 9, as has been described for the four sets of four compounds: 4, 8, 13, 19; 5, 9, 15, 20; 6, 9, 17, 21; and 7, 10, 18 and 22. The only anomaly is that arising in the spectrum of compound 1 (biliverdin III α), which has been described under 'Biliverdins', presumably owing to the planar U-shaped molecule in the verdins.

*Violins with an ethylidene group in the unconjugated end ring, R*R* compounds.* Some of the signals common to compounds 23, 24, 27 and 28 have been observed in previously described violin spectra (Stoll & Gray, 1970). These signals (Fig. 7 and Table 5) are a doublet at about 1.05 (3 protons), a quartet at about 1.74 (3 protons), an obscured multiplet at 2.10 (1 proton), a partly obscured octet centred at 2.98 (2 protons) and multiplets at 4.64 (1 proton) and 5.30 (1 proton). The positions of all these coupled signals

Fig. 5. Fourier-transform n.m.r. spectra at 90 MHz in C^2HCl_3 of dihydrobiliviolins, (a) 'exo'-dihydrobiliviolin III α dimethyl ester, (b) A-dihydroisobiliviolin IX α dimethyl ester, (c) D-dihydrobiliviolin IX α dimethyl ester and (d) 'exo'-dihydrobiliviolin XIII α dimethyl ester

Me_4Si , tetramethylsilane. Each proton, or group of equivalent protons, in the molecule is numbered in the structures shown. N.m.r. signals arising from these protons are indicated in the spectrum by the appropriate proton number. The total width of multiplet signals is indicated by horizontal brackets.

and their interrelations were discovered by spin-decoupling experiments performed on compound 27, which elucidated the structure (Fig. 8). The spin-decoupling results can be explained by the presence of an unconjugated pyrrolidone end ring bearing an ethylidene substituent at the 3-position and a methyl substituent at the 2-position (Structure I, Fig. 8). The methyl protons (b) of the ethylidene group are coupled strongly to proton A and weakly to proton C, homoallylic to them. This methyl signal is therefore a quartet centred at 1.74, the wide spacing (7 Hz) being due to the effect of proton A and the narrow spacing (4 Hz) to the effect of proton C. Irradiation at 5.30 (proton A resonance) caused the quartet due to protons (b) to collapse to a close doublet of spacing 4 Hz, and irradiation at 2.10 (proton C resonance) gave a wide doublet of spacing 7 Hz. Proton C is strongly coupled also to the protons of the secondary methyl group (c), which therefore appears as a doublet centred at 1.06 of wide spacing (7 Hz), collapsing to a singlet during irradiation at 2.10. Proton A is weakly coupled to proton C (allylic to it), and during irradiation at 2.10 the multiplet due to proton A collapses to a quartet of spacing 7 Hz. The geminal methylene protons (a) are non-equivalent, because of the adjacent asymmetric centre, to which proton B is attached. They are strongly mutually coupled and weakly coupled also to proton B, and therefore appear in the spectra as an octet centred at 2.98, which

is partly obscured by the signals at 2.88 from the 8¹ and 12¹ protons of the methoxycarbonyl ethyl substituents. The octet has a wide spacing (14 Hz) due to mutual geminal coupling, and a narrow spacing (4 Hz) due to the effect of proton B. Irradiation at 4.64 (proton B resonance) caused the octet due to (a) protons to collapse to a quartet of spacing 14 Hz. Structure I is more probable than structure II, because irradiation at 2.10 (proton C resonance) had no effect on the multiplet due to proton B showing no coupling between B and C because of the distance between them. In structure II (Fig. 8), however, coupling would be expected because of the proximity of B and C protons.

The lack of coupling between proton B and the ethylidene methyl protons (b), however, is regarded as evidence that the latter is *trans* to the inner bond of the pyrrolidone ring, since *trans*-homoallylic coupling constants are known to be much smaller than *cis*-coupling constants (Jackman & Sternhell, 1969).

Spin-decoupling experiments have shown the position of the signal from the 18¹ protons of the 18-ethyl substituent in compound 27. Irradiation at 2.39 caused the triplet at 1.13 to collapse to a singlet; thus 2.39 is the origin position of the quartet corresponding to the methylene protons.

Further evidence that the ethylidene group is in the inner position of the unconjugated end ring is seen in comparison of the spectra of the ethylidene-contain-

Fig. 6. Fourier-transform n.m.r. spectra at 90 MHz in C^2HCl_3 of biliviolins and a dihydrobiliviolin, (a) biliviolin III α dimethyl ester, (b) biliviolin IX α dimethyl ester and (c) A-dihydrobiliviolin IX α dimethyl ester Me_4Si , tetramethylsilane. Each proton, or group of equivalent protons, in the molecule is numbered in the structures shown. N.m.r. signals arising from these protons are indicated in the spectrum by the appropriate proton number. The total width of multiplet signals is indicated by horizontal brackets.

ing violins 27, 28, 23 and 24 with those of the corresponding ethyl-containing violins 20, 22, 15 and 17 respectively.

An upfield shift of the methyl signal at the inner end (position 7 or 13) of the chromophore is observed in the spectra of compounds 27, 28, 23 and 24 (1.89 in all cases) compared with those of compounds 20, 22, 15 and 18 (1.98 in all cases). Construction of a model shows the proximity of the ethylidene double bond with its shielding effect on protons in an axial position relative to it.

Comparison of the unconjugated end rings in these two groups of compounds is equivalent to a system in which the double bond in a five-carbon chain moves from the 2,3- to the 3,4-position. Thus, as shown below, C-5 in structure A is chemically equivalent to C-1 in structure B, and C-1 in structure A is chemically equivalent to C-5 in structure B:

Structure A

Structure B

The situation in the violins under study is more complex, but the same general principle applies, thus:

The positions of the signals from the methyl group of the ethyl group in the unconjugated end ring in the spectra of compounds 20, 22, 15 and 18, at 1.07, 1.06, 1.07 and 1.07 respectively, correspond closely to the

signals from the secondary methyl group in the unconjugated end ring in the spectra of compounds 27, 28, 23 and 24 at 1.06, 1.05, 1.05 and 1.05 respectively. Similarly, the positions of the signals from the methyl group in the unconjugated end ring in the spectra of compounds 20, 22, 15 and 18, at 1.70, 1.69, 1.70 and 1.69 respectively, correspond closely to the signals from the methyl group of the ethylidene group in the spectra of compounds 27, 28, 23 and 24 at 1.74, 1.74, 1.73 and 1.74 respectively.

The position of the signal from the 16 or 4 proton of the unconjugated end ring in the spectra of the '*R***R**-ethylidene'-violins is downfield at 4.66 to 4.62 compared with its position in the spectra of the 'normal'-violins at 4.41 to 4.38. This must be due to the deshielding effect of the ethylidene double bond on the equatorially placed 16 or 4 proton.

These observations show conclusively that the ethylidene group is in the inner position of the unconjugated end ring.

There is a similar relationship between compounds 20 and 27, 22 and 28, 15 and 23 and 18 and 24. The members of each pair of compounds differ only in the position of the double bond in the unconjugated end ring as described. Therefore compound 27 is

'*R***R**-ethylidene'-isomesobiliviolin IX α , compound 28 is '*R***R**-ethylidene'-mesobiliviolin XIII α , compound 23 is '*R***R**-ethylidene'-dihydroisobiliviolin IX α and compound 24 is '*R***R**-ethylidene'-dihydro-

Fig. 7. Fourier-transform n.m.r. spectra at 90 MHz in C_2HCl_3 of '*R*R*'-ethylidene'-violins, (a) '*R*R*'-ethylidene'-dihydroisobiliviolin IX α dimethyl ester, (b) '*R*R*'-ethylidene'-dihydrobiliviolin XIII α dimethyl ester, (c) '*R*R*'-ethylidene'-isomesobiliviolin IX α dimethyl ester and (d) '*R*R*'-ethylidene'-mesobiliviolin XIII α dimethyl ester

Me_4Si , tetramethylsilane. Each proton, or group of equivalent protons, in the molecule is numbered in the structures shown. N.m.r. signals arising from these protons are indicated in the spectrum by the appropriate proton number. The total width of multiplet signals is indicated by horizontal brackets.

biliviolin XIII α . The relative stereochemistry of the two asymmetric centres was established by a comparative study with their diastereoisomeric '*R*S*'-ethylidene'-violins described below.

*Violins with an ethylidene group in the unconjugated end ring, *R*S* compounds.* Base-catalysed isomerization of compounds 27, 28, 23 and 24 (the '*R*R*'-ethylidene'-violins) gave compounds 20, 22, 15 and 18 (the 'normal'-violins) respectively, with small amounts of compounds 29, 30, 25 and 26 (the '*R*S*'-ethylidene'-violins) respectively. Mass spectrometry showed them to be isomeric with the corresponding '*R*R*'-ethylidene'- and 'normal'-violins and to contain corresponding chromophores; this was fully supported by the electronic spectroscopy. The n.m.r. spectra of the '*R*S*'-ethylidene'-violins 25, 26, 29 and 30 (Fig. 9 and Table 5) are identical with those of the corresponding '*R*R*'-ethylidene'-violins 27, 28, 23 and 24 respectively, except for the following.

(a) A large upfield shift of the doublet signal at 1.06 to 1.05 from the secondary methyl group of the unconjugated end ring to an abnormally high-field position at 0.64 to 0.62 for a secondary methyl group (position 2¹ or 18¹).

(b) A decrease in the coupling constant of the methyl protons of the ethylidene methyl group to the tertiary 2 or 18 proton of the unconjugated end ring. Thus the doublet signal of the ethylidene methyl group in the spectra of the '*R*R*'-ethylidene'-violins becomes a broad doublet in those of the '*R*S*'-

ethylidene'-violins, but undergoes no change in position.

(c) Small shifts of some signals described below.

The quantity of the most abundant '*R*S*'-ethylidene'-violin (compound 29) was insufficient for spin decoupling, but it was expected that a shift in the position of the signal from the tertiary 2 or 18 proton of the unconjugated end ring would be observed.

These changes are explained by an inversion of configuration of the outer 2 or 18 carbon atom of the unconjugated end ring via base-catalysed enolization (see under 'Base-catalysed isomerization').

Because the '*R*R*'-ethylidene'-violins contain two asymmetric centres, the inner 4 or 16 carbon atoms and the outer 2 or 18 carbon atoms of the unconjugated end ring, two racemic diastereoisomers are possible. Models have shown that in the relative configuration proposed for the '*R*R*'-ethylidene'-violins the secondary methyl group lies over the adjacent pyrrole ring and is thus strongly shielded by it, explaining the high-field position of its n.m.r. signal in these isomers (A. H. Jackson, personal communication). Inversion of one centre only would convert one diastereoisomer into the other, and, since diastereoisomers differ in chemical and physical properties, they were separable by t.l.c., as has been observed in the urobilins (Stoll & Gray, 1970). The '*R*R*'-ethylidene'-violins represent one diastereoisomeric form in which the relative configurations of the two asymmetric centres are fixed, and the '*R*S*'-

Fig. 8. Spin-decoupling experiments performed on compound 27

ethylidene'-violins represent the other diastereoisomeric form in which the relative configurations of the two asymmetric centres are opposite to those in the '*R***R**-ethylidene'-violins. This racemization of the secondary methyl group is further evidence that this group is in the outer position of the unconjugated ring, next to the enolizable carbonyl group. This is further supported by the chemical properties of the '*R***S**-ethylidene'-violins; thus they resist FeCl_3 dehydrogenation, and isomerize readily to the corresponding 'normal'-violin with a small amount of the corresponding '*R***R**-ethylidene'-violin. Other possible structures for compounds 25, 26, 29 and 30 include double-bond isomers of the '*R***R**-ethylidene'- and 'normal'-violins. Thus they might have contained the unconjugated rings shown in structures (1)–(4).

The n.m.r. data did not support any of these structures. Structure 3 contains an ethyl group and no secondary methyl group, both of which are inconsistent with the data. Structures 2 and 4 both contain secondary methyl groups, but these structures are impossible because they contain ethyl groups. Structure 1 is equally impossible, because, although it contains a secondary methyl group, it has a vinyl group for which there is no n.m.r. evidence. Structure 3 may also be discounted on chemical grounds, because 'normal'-violins do not isomerize to '*R***R**-ethylidene'-violins; there is no mechanism for isomerization of a compound containing structure 3 to an '*R***R**-ethylidene'-violin without invoking a 'normal'-violin as an intermediate. Finally, the n.m.r. data show clearly that the '*R***S**-ethylidene'-violins contain an ethylidene group and that the 16 or 4 proton is present and not coupled in a significantly different way from that in the '*R***R**-ethylidene'-violins. In structures 2 and 4 the 16 or 4 proton is absent and in structures 1 and 3 its coupling to the inner 17 or 3 proton would profoundly alter its n.m.r. signal.

Changes in the chemical shifts of protons attached to the unconjugated end ring further reflect the change in geometry of the system, with inversion of the relative configuration of its two asymmetric centres, i.e. signals from the 16 or 4 protons, the NH proton and the tertiary proton of the ethylidene group lie at 4.66–4.62, 8.58–8.51 and 5.32–5.30 respectively in the spectra of compounds 23, 24, 27 and 28, and at 4.74–4.72, 8.21–8.06 and 5.41–5.40 respectively in the spectra of compounds 25, 26, 29 and 30.

Electronic spectroscopy

The electronic-spectroscopic data for the ten verdin dimethyl esters are shown in Table 6. Significant shifts in the position of the maxima and minima, in the relative intensities of the u.v. and visible maxima and

in the appearance of the absorption curve only occur with a change in conjugation of the verdin chromophore, i.e. the isomeric glaucobilins had almost identical spectra each having a similar chromophore without external double bonds (mean λ_{max} at 369 and 631 nm and mean $\epsilon_{369}/\epsilon_{631}$ of 3.5). The isomeric biliverdins each showed markedly different spectra, since each has a different chromophore, i.e. biliverdin III α with two linearly conjugated vinyl groups has λ_{max} at 381 and 661.5 nm, biliverdin IX α with one linearly conjugated vinyl group and one cross-conjugated vinyl group has λ_{max} at 380 and 657 nm, whereas biliverdin XIII α with two cross-conjugated vinyl groups has λ_{max} at 378 and 648 nm. The spectra of the four dihydrobiliverdins fell into two groups: the spectra of dihydrobiliverdin III α and A-dihydrobiliverdin IX α were similar, having a chromophore each with one linearly conjugated vinyl group (mean λ_{max} at 376 and 649 nm and mean $\epsilon_{376}/\epsilon_{649}$ of 4.0), whereas the spectra of D-dihydrobiliverdin IX α and dihydrobiliverdin XIII α were similar, each having a chromophore with one cross-conjugated vinyl group (mean λ_{max} at 374 and 647 nm and mean $\epsilon_{374}/\epsilon_{647}$ of 3.2, due to a decrease in the mean u.v. extinction coefficient of 20% compared with the previous group).

The electronic spectra of the 19 violin dimethyl esters (Table 6) fell into three major groups, corresponding to three different chromophores: type A, dihydrobiliviolins and biliviolins containing a linearly conjugated double bond attached to the chromophore; type B, dihydrobiliviolins and biliviolins containing one cross-conjugated double bond attached to the chromophore; type C, the mesobiliviolins without vinyl groups and a dihydrobiliviolin containing one vinyl group in the unconjugated end ring, i.e. compounds having a violin chromophore with no additional double bonds. Type A violins showed maxima of 341–342 nm and 581–587 nm; maxima for type B were 336–338 nm and 576–582 nm and those for type C 329–330 nm and 564–571 nm. Changes in the structure of the unconjugated end ring and in the position of saturated substituents attached to the chromophore had little effect on the electronic spectra. However, the '*R***R**-ethylidene' compounds 23, 24, 27 and 28 and '*R***S**-ethylidene' compounds 25, 26, 29 and 30 showed bathochromic shifts of about 5 and 2 nm respectively in their visible maxima relative to the other compounds containing the same chromophore.

A special effect of the vinyl substituents was observed in both the verdin and the violin spectra. Outer vinyl substituents, which were therefore linearly conjugated, produced a mean increase in extinction coefficient of 24 and 6% in u.v. and visible maxima respectively and an increase in the $\epsilon^{\text{u.v.}}/\epsilon^{\text{vis.}}$ ratio of 15% compared with a corresponding tripyrrolic chromophore without a vinyl substituent; an inner

Fig. 9. Fourier-transform n.m.r. spectra at 90 MHz in C^2HCl_3 of 'R*S*-ethylidene'-violins, (a) 'R*S*-ethylidene'-dihydroisobiliviolin IX α dimethyl ester, (b) 'R*S*-ethylidene'-dihydrobiliviolin XIII α dimethyl ester, (c) 'R*S*-ethylidene'-isomesobiliviolin IX α dimethyl ester and (d) 'R*S*-ethylidene'-mesobiliviolin XIII α dimethyl ester

Me_4Si , tetramethylsilane. Each proton, or group of equivalent protons, in the molecule is numbered in the structures shown. N.m.r. signals arising from these protons are indicated in the spectrum by the appropriate proton number. The total width of multiplet signals is indicated by horizontal brackets.

vinyl substituent which was therefore cross-conjugated produced a decrease in extinction coefficient of 24 and 6% in u.v. and visible maxima respectively and a decrease of 18% in the $\epsilon^{u.v.}/\epsilon^{vis.}$ ratio compared with a corresponding chromophore without a vinyl substituent.

The electronic spectroscopy thus supports the structural assignments for the compounds.

Hydrogenation experiments

Hydrogenation of violins and verdins in formic acid by using palladized charcoal as catalyst converted vinyl into ethyl groups, but produced no hydrogenation of the chromophore under the conditions used. The ethylidene moiety was stable under these conditions. All vinyl compounds studied were converted into the corresponding meso compounds in this way,

Table 6. *Electronic-spectral data for the pigment dimethyl esters in chloroform*

Each value is the mean of three separate determinations. $\lambda_{\text{max}}^{\text{u.v.}}$ (nm), $\lambda_{\text{max}}^{\text{vis.}}$ (nm) and $\lambda_{\text{min.}}$ (nm) are the wavelengths of the u.v.- and visible-absorption maxima and the absorption minima respectively. $\epsilon^{\text{u.v.}}$, $\epsilon^{\text{vis.}}$, $\epsilon^{\text{min.}}$ are the molar extinction coefficients of the u.v.- and visible-absorption maxima and the absorption minima respectively.

Compound type	Compound no.	Wavelengths (nm)			Extinction coefficients (litre·mol ⁻¹ ·cm ⁻¹)					
		$\lambda_{\text{max}}^{\text{u.v.}}$	$\lambda_{\text{min.}}$	$\lambda_{\text{max}}^{\text{vis.}}$	$\epsilon^{\text{u.v.}}$	$\epsilon^{\text{min.}}$	$\epsilon^{\text{vis.}}$	$(\epsilon^{\text{u.v.}}/\epsilon^{\text{vis.}})$	$(\epsilon^{\text{vis.}}/\epsilon^{\text{min.}})$	$(\epsilon^{\text{u.v.}}/\epsilon^{\text{min.}})$
Verdin	1	381	495	661.5	68000	2020	14200	4.77	7.05	33.6
	2	380	489	657	55700	2270	15500	3.59	6.83	24.5
	3	378	487	648	44900	2290	15700	2.85	6.87	19.6
	4	376.5	484	648	60400	1840	14700	4.12	7.97	32.8
	5	376	485	649	57900	1910	14900	3.89	7.79	30.3
	6	374	480	647	48400	1470	15000	3.23	10.2	33.0
	7	373	481	646	46800	1710	15200	3.07	9.17	28.2
	8	369	473	631	55300	1750	15800	3.50	9.01	31.5
	9	369	473	631	54700	1690	15800	3.46	9.35	32.4
	10	368	471	631.5	53200	1750	15500	3.43	8.85	30.4
Type A violin	11	341.5	439	581	45000	3040	27800	1.62	9.16	14.8
	13	342	440	583	45900	3030	29000	1.58	9.57	15.2
	15	342	443	582	45200	3180	27800	1.63	8.74	14.2
	23	342	442	587	43000	2900	27900	1.54	9.59	14.8
	25	341	444	585	40600	2950	25600	1.59	8.69	13.8
Type B violin	12	337	444	576	—	—	—	1.19	7.05	8.38
	17	337.5	440	579	28600	3090	26300	1.09	8.51	9.25
	18	336	434	577	27100	2730	24800	1.09	9.10	9.90
	24	338	448	582	25900	2940	22900	1.13	7.78	8.81
	26	337.5	437	580	27000	2700	23700	1.14	8.75	9.99
Type C violin	14	Not investigated								
	19	329	422	567	35000	2670	25200	1.39	7.99	11.7
	20	329	420	564	38800	2590	27400	1.42	10.6	15.0
	27	330	434	571	35400	2990	27000	1.31	9.01	11.8
	29	329.5	424	569	37200	2640	27100	1.37	10.3	14.1
	16	329.5	442	566	31000	3830	21700	1.43	5.66	8.09
	21	329	438.5	566.5	35600	2850	25700	1.39	8.99	12.5
	22	328.5	423	566	36500	2490	26600	1.37	10.7	14.7
	28	329.5	425	571	38300	2830	28200	1.36	9.97	13.5
	30	329	423	568	35900	2450	25600	1.40	10.4	14.6

thus demonstrating interrelationships between the new compounds and those previously characterized. The results of these hydrogenation experiments are shown in Table 7. All attempts to convert biliverdins into dihydrobiliverdins and biliviols into dihydrobiliviols failed, presumably because the dihydro intermediates could not accumulate under these conditions, but were rapidly further hydrogenated to the meso end products. The hydrogenation products were identified by their colour and mobility in t.l.c. systems 1–7 (Table 8); meso compounds were used as internal standards.

The expected products were obtained in every case. T.l.c. separation of '*R***S**-ethylidene'-isomobiliviolin IX α dimethyl ester (compound 29) and '*R***S**-ethylidene'-mesobiliviolin XIII α dimethyl ester (compound 30) was not accomplished, and thus, for studying compounds 25 and 26 by hydrogenation, base-catalysed isomerization converted compound 25

into 15 and compound 26 into 18, and subsequent hydrogenation of compound 15 to 20 and compound 18 to 22. Compounds 20 and 22 were then unambiguously identified by t.l.c.

Dehydrogenation with FeCl₃

All 19 violins were treated with FeCl₃ under standard conditions for dehydrogenation, and the products identified by t.l.c. by using the ten verdins as internal standards (Table 9). Every violin gave the expected corresponding verdin, except (a) the '*R***R**-ethylidene'- and '*R***S**-ethylidene'-violins, which under these conditions were stable and gave no verdin at all, and (b) those containing a vinyl group in the unconjugated end ring, which gave unidentified verdins of low chromatographic mobility; these were probably formed by methanol addition to the vinyl group in the unconjugated end ring and possibly

Table 7. *Products of catalytic hydrogenation, with palladized charcoal in formic acid, of pigment dimethyl esters*

Products were identified by t.l.c. by using standards for comparison. The compound number of the starting material and that of its major hydrogenation product are shown. The compounds are as follows. (1) Biliverdin III α ; (2) biliverdin IX α ; (3) biliverdin XIII α ; (4) dihydrobiliverdin III α ; (5) A-dihydrobiliverdin IX α ; (6) D-dihydrobiliverdin IX α ; (7) dihydrobiliverdin XIII α ; (8) mesobiliverdin III α ; (9) mesobiliverdin IX α ; (10) mesobiliverdin XIII α ; (11) biliviolin III α ; (12) biliviolin IX α ; (13) 'exo'-dihydrobiliviolin III α ; (14) 'endo'-dihydrobiliviolin III α ; (15) A-dihydroisobiliviolin IX α ; (16) A-dihydrobiliviolin IX α ; (17) D-dihydrobiliviolin IX α ; (18) 'exo'-dihydrobiliviolin XIII α ; (19) mesobiliviolin III α ; (20) isomesobiliviolin IX α ; (21) mesobiliviolin IX α ; (22) mesobiliviolin XIII α ; (23) 'R*R*-ethylidene'-dihydroisobiliviolin IX α ; (24) 'R*R*-ethylidene'-dihydrobiliviolin XIII α ; (27) 'R*R*-ethylidene'-isomesobiliviolin IX α ; (28) 'R*R*-ethylidene'-mesobiliviolin XIII α .

Compound no.	Compound no. of major product
1	8
2	9
3	10
4	8
5	9
6	9
7	10
11	19
12	21
13	19
14	Not investigated
15	20
16	19
17	19
18	22
23	27
24	28

also to the outer vinyl group in the chromophore in biliviolin III α dimethyl ester. These experiments further demonstrated the relationship between the new compounds.

'R*R*-Ethylidene' and 'R*S*-ethylidene' compounds failed to undergo dehydrogenation with FeCl₃ to compounds of the phycocyanobilin type, in which the bridge to the ring containing the ethylidene substituent is unsaturated (Chapman *et al.*, 1967; Cole *et al.*, 1967). This is probably because the bonds of the α -carbon atom (position 4 or 16) to the methylene-bridge carbon atom (position 5 or 15) and the α -proton (position 4 or 16) are not polarized by conjugation with the carbonyl group via a ring double bond as they are in the 'normal'-violins (Scheme 5).

No way has yet been found to dehydrogenate this

bond to yield phycocyanobilin-type compounds, but attempts to achieve this will continue, as this represents a route from bilirubin to this class of natural bile pigments.

A similar stability towards FeCl₃ is shown by stercobilin and violins containing a fully hydrogenated end ring (Watson *et al.*, 1969; Petryka *et al.*, 1968), which also have no polarization of the appropriate bonds.

Base-catalysed isomerization

Each compound was treated with methanolic sodium methoxide under standard conditions. All verdins and 'normal'-violins were stable, except those containing a vinyl group in an unconjugated end ring, which gave several unidentified products including verdins.

Each 'R*R*-ethylidene'- and 'R*S*-ethylidene'-violin consistently gave, without significant side reactions, products which were identified by t.l.c. by using internal standards (Table 10). The migration of an ethylidene double bond into an end ring of a bile pigment under base catalysis has been reported before (Chapman *et al.*, 1967; Cole *et al.*, 1967).

The 'R*R*-ethylidene'- or 'R*S*-ethylidene'-violins gave, after brief treatment with methanolic sodium methoxide, small quantities of the corresponding 'R*S*-ethylidene'- or 'R*R*-ethylidene'-violins respectively and 'normal'-violins. Treatment with base for longer periods resulted in no appreciable change in the yield of diastereoisomeric ethylidene-containing violin, but a large increase in the yield of 'normal'-violin. For this reason, appreciable yields of 'R*S*-ethylidene'-violins from 'R*R*-ethylidene'-violins could be achieved only by a succession of brief base treatments followed each time by isolation by t.l.c. of the products and recycling of the starting material (see the Experimental section). The 'normal'-violins obtained in these experiments were identified also by FeCl₃ dehydrogenation to the corresponding verdin, which was in turn identified by t.l.c. by using internal standards.

That base-catalysed isomerization of 'R*R*-ethylidene'- and 'R*S*-ethylidene'-violins resulted in their interconversion and conversion into 'normal'-violins lends support to the assertion that the ethylidene group is in the inner position of the unconjugated end ring, because (a) inversion of the β -carbon atom via keto-enol tautomerism is possible only if the secondary methyl group is in the outer β -position of the unconjugated end ring adjacent to the carbonyl group and (b) the driving force of increased conjugation by conversion of 'ethylidene'- into 'normal'-violin only applies with an inner ethylidene group, since an outer one would be already so conjugated.

Table 8. *Thin-layer-chromatographic data for the pigment dimethyl esters in six solvent systems*

Values are R_M relative to compound 9 as internal standard ($n = 30$). Mean R_F with standard deviation is given for compound 9. Chromatography was performed in equilibrated tanks on non-equilibrated plates at room temperature with a development distance of 16cm.

Compound no.	Solvent system 1	Solvent system 2	Solvent system 4	Solvent system 5	Solvent system 6	Solvent system 7
1	1.28	1.24	1.20	1.13	1.09	1.04
2	1.13	1.14	1.08	1.06	1.04	1.00
3	1.00	1.00	1.00	1.00	1.00	1.00
4	1.20	1.15	1.11	1.09	1.08	1.03
5	1.08	1.11	1.05	1.04	1.03	1.02
6	1.00	1.05	1.03	1.03	1.00	1.00
7	0.93	0.96	0.97	1.00	0.96	1.00
8	1.05	1.07	1.06	1.04	1.03	1.00
R_F 9	0.259	0.353	0.395	0.515	0.445	0.535
S.D. ($n = 30$)	0.013	0.017	0.018	0.020	0.021	0.21
10	0.89	0.87	0.92	0.95	0.93	0.97
11	0.56	0.82	0.69	0.91	0.77	0.98
12	0.40	0.64	0.51	0.72	0.64	0.86
13	0.35	0.59	0.38	0.67	0.51	0.72
14	Pure compound not investigated because not separated from compound 28					
15	0.29	0.48	0.39	0.63	0.43	0.70
16	0.39	0.62	0.49	0.76	0.66	0.85
17	0.23	0.42	0.33	0.58	0.36	0.65
18	0.21	0.37	0.30	0.52	0.33	0.61
19	0.27	0.43	0.35	0.60	0.47	0.71
20	0.21	0.36	0.32	0.55	0.37	0.63
21	0.25	0.40	0.30	0.55	0.43	0.68
22	0.18	0.33	0.26	0.49	0.29	0.59
23	0.67	0.94	0.76	0.96	0.81	0.95
24	0.50	0.72	0.60	0.82	0.61	0.87
25	0.45	0.70	0.53	0.78	0.64	0.83
26	0.30	0.50	0.38	0.63	0.47	0.73
27	0.52	0.75	0.65	0.87	0.68	0.89
28	0.48	0.71	0.58	0.83	0.62	0.87
29	0.33	0.53	0.42	0.67	0.48	0.74
30	0.35	0.52	0.40	0.66	0.51	0.76

Scheme 5. Failure of $FeCl_3$ to dehydrogenate ethylidene compounds to a phycocyanobilin, and dehydrogenation by $FeCl_3$ of normal violin to a phycocyanobilin

Table 9. *FeCl₃ dehydrogenation products of pigment dimethyl esters*

Products were identified by t.l.c. by using standards for comparison. The compound number of the starting material and those of its major dehydrogenation product(s) are shown. The compounds are as follows. (2) Biliverdin IX α ; (4) dihydrobiliverdin III α ; (5) A-dihydrobiliverdin IX α ; (6) D-dihydrobiliverdin IX α ; (7) dihydrobiliverdin XIII α ; (8) mesobiliverdin III α ; (9) mesobiliverdin IX α ; (10) mesobiliverdin XIII α ; (11) biliviolin III α ; (12) biliviolin IX α ; (13) 'exo'-dihydrobiliviolin III α ; (14) 'endo'-dihydrobiliviolin III α ; (15) A-dihydroisobiliviolin IX α ; (16) A-dihydrobiliviolin IX α ; (17) D-dihydrobiliviolin IX α ; (18) 'exo'-dihydrobiliviolin XIII α ; (19) mesobiliviolin III α ; (20) isomesobiliviolin IX α ; (21) mesobiliviolin IX α ; (22) mesobiliviolin XIII α . No product was obtained from compounds 23–30.

Compound no.	Compound no. of dehydrogenation product
11	Unidentified verdins
12	2+Unidentified verdins
13	4
14	Unidentified verdins
15	5
16	5+Unidentified verdins
17	6
18	7
19	8
20	9
21	9
22	10

Chromic acid oxidation

Each compound was oxidized with chromic acid. The imides so obtained were identified by g.l.c. rather than by t.l.c., with the carcinogenic *o*-tolidine as colour reagent. The results were as expected, except that the 'R*R*-ethylidene'- and 'R*S*-ethylidene'-violins appeared to give ethylmethylmaleimide but no ethyldenemethylsuccinimide. Ethylmethylmaleimide was detected for all compounds except 1, 2, 3, 11 and 12; methylvinylmaleimide was detected in compounds 1–7, 11, 12, 13, 15, 16–18 and 23–26. In no case was ethyldenemethylsuccinimide detected. Compound 14 was not investigated. Attempts to identify this imide by high-pressure liquid chromatography, which avoids the use of high temperatures, were also without success. The imide may have been formed, but was not detected by the methods used; the conditions of oxidation probably resulted in the isomerization of the ethylidene group into the ring, giving ethylmethylmaleimide.

Chromic acid oxidation did not support the proposed structures of the 'R*R*-ethylidene'- and 'R*S*-ethylidene'-violins, but because compounds of similar structure have not been previously

Table 10. *Products of base-catalysed isomerization of 'R*R*-ethylidene'- and 'R*S*-ethylidene'-violins*

Products were identified by t.l.c. by using standards for comparison. The compound number of the starting material and that of its major products are shown. The compounds are: (15) A-dihydroisobiliviolin IX α ; (18) 'exo'-dihydrobiliviolin XIII α ; (20) isomesobiliviolin IX α ; (22) mesobiliviolin XIII α ; (23) 'R*R*-ethylidene'-dihydroisobiliviolin IX α ; (24) 'R*R*-ethylidene'-dihydrobiliviolin XIII α ; (25) 'R*S*-ethylidene'-dihydroisobiliviolin IX α ; (26) 'R*S*-ethylidene'-dihydrobiliviolin XIII α ; (27) 'R*R*-ethylidene'-isomesobiliviolin IX α ; (28) 'R*R*-ethylidene'-mesobiliviolin XIII α ; (29) 'R*S*-ethylidene'-isomesobiliviolin IX α ; (30) 'R*S*-ethylidene'-mesobiliviolin XIII α .

Compound no.	Compound no. of isomerization products
23	25+15
24	26+18
25	23+15
26	24+18
27	29+20
28	30+22
29	27+20
30	28+22

described little can be concluded from this. Rather, the experiments show the possible error of assuming similar chemical behaviour from compounds of only approximately similar structure, e.g. phycoerythrobilin and phycocyanobilin, each of which contains a dehydrogenated outer bridge in the part structure:

compared with the hydrogenated outer bridge in the part structure proposed for the 'R*R*-ethylidene'- and 'R*S*-ethylidene'-violins.

Phycoerythrobilin and phycocyanobilin gave ethyldenemethylsuccinimide on chromic acid oxidation (Rüdiger *et al.*, 1967).

Central dipyrromethane structure
common to all pigments

End rings present in
violins and verdins (group A)

End rings present only in violins (Group B)

Scheme 6. Structures of compounds formed by rearrangement

Combinations of left-hand rings gives ten verdins. Combinations of left-hand with right-hand rings gives 20 violins.

Thin-layer chromatography

The chromatographic properties in six solvent systems of the 29 compounds are shown in Table 8. Since absolute R_F values are sensitive to small changes in condition of the t.l.c. plate, temperature and vapour-equilibration conditions, R_F values are expressed relative to that of glaucobilin IX α dimethyl

ester, which was always run as an internal standard (R_M values). The R_F of this marker compound is given in each case also.

Certain trends relating structure to mobility can be discerned.

(a) All verdins studied are more mobile than all violins studied.

(b) If all other structural features are similar mobilities were in the order $\text{III}\alpha > \text{IX}\alpha > \text{XIII}\alpha$.

(c) If all other structural features are similar, mobilities were in the order 'R*R*-ethylidene'-violin > violin with vinyl group in unconjugated end ring > 'R*S*-ethylidene'-violin > 'normal'-violin.

(d) If all other structural features are similar, mobilities were in the order violins containing an outer vinyl group in the chromophore > violins containing an inner vinyl group in the chromophore > violins with no vinyl group in the chromophore.

Conclusion

All the compounds that have been synthesized and characterized have a common central dipyrromethene structure, but differ in the nature of the two end rings and include: (1) biliverdins and biliviols, containing two vinyl substituents; (2) dihydrobiliverdins with one vinyl and one ethyl substituent; (3) dihydrobiliviols with one vinyl substituent either inside or outside the chromophore, and either one ethyl or one ethylidene substituent, the latter always in the outer position of an unconjugated end ring occurring in two diastereoisomeric forms; (4) mesobiliverdins with two ethyl substituents; (5) mesobiliviols with one ethyl substituent inside the chromophore and additionally one ethyl or one ethylidene substituent as in the dihydrobiliviols.

The structures of the ten verdins are formally derived by combining the four end-ring part structures from Group A (Scheme 6) including duplication, with the central dipyrromethene, and the structures of the 20 violins by combining one end-ring part structure from Group A and one end-ring part structure from Group B (Scheme 6) with the central dipyrromethene.

A future study could involve reduction of the central-bridge double bond of all the compounds, probably with NaBH_4 , to produce the corresponding rubins, which may be of greater biological interest.

The availability of these model compounds and their electronic, mass, and n.m.r. spectra should be of value in the elucidation of bile pigments isolated from natural sources.

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References

- Bonnett, R. & McDonagh, A. F. (1970) *J. Chem. Soc. D* 238–239
- Chapman, D. J., Cole, W. J. & Siegelman, H. W. (1967) *J. Am. Chem. Soc.* **89**, 5976–5977
- Cole, W. J., Chapman, D. J. & Siegelman, H. W. (1967) *J. Am. Chem. Soc.* **89**, 3643–3645
- Cooper, G., Games, D. E., Jackson, A. H., Saxton, R. G. & Stoll, M. S. (1975) in *Advances in Mass Spectrometry in Biochemistry and Medicine* (Frigerio, A., ed.), vol. 2, Spectrum Publications, New York, in the press
- Fischer, H. & Haberland, H. W. (1935) *Hoppe-Seyler's Z. Physiol. Chem.* **232**, 236–258
- Jackman, L. M. & Sternhell, S. (1969) *Applications of NMR Spectroscopy in Organic Chemistry*, 2nd edn., pp. 316–330, Pergamon Press, Oxford
- Jansen, F. H. & Stoll, M. S. (1971) *Biochem. J.* **125**, 585–597
- McDonagh, A. F. & Assisi, F. (1971) *FEBS Lett.* **18**, 315–317
- McDonagh, A. F. & Assisi, F. (1972) *J. Chem. Soc. Chem. Commun.* **3**, 117–119
- Ó Carra, P. & Colleran, E. (1970) *J. Chromatogr.* **50**, 458–468
- Pearson, L. B. & Watson, C. J. (1963) *Proc. Soc. Exp. Biol. Med.* **112**, 756–758
- Petryka, Z. J., Watson, C. J., Davis, E., Weimer, M., Lightner, D. & Moscovitz, A. (1968) *Tetrahedron Lett.* **57**, 5983–5986
- Rüdiger, W. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1291–1300
- Rüdiger, W., Ó Carra, P. & Ó hEocha, C. (1967) *Nature (London)* **215**, 1477–1478
- Stoll, M. S. & Gray, C. H. (1970) *Biochem. J.* **117**, 271–290
- Watson, C. J., Weimer, M., Petryka, Z. J., Lightner, D. A., Moscovitz, A., Davis, E. & Beach, N. A. (1969) *Arch. Biochem. Biophys.* **131**, 414–422