Biochem. J. (1973) **133**, 493–498
Printed in Great Britain

The Synthesis of Esters of Bilirubin

By DAVID W. HUTCHINSON,* BRIAN JOHNSON and ALAN J. KNELL
Department of Molecular Sciences, University of Warwick, Coventry CV4 7AL, U.K.

(*Received* 4 *December* 1972)

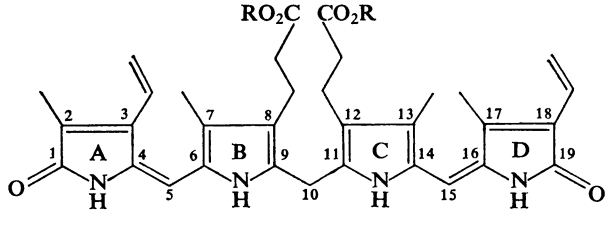
1-Alkyl-3-*p*-tolyltriazenes were used to synthesize the methyl, ethyl, isopropyl and benzyl esters of bilirubin. Treatment of a chloroform solution of bilirubin with the triazene at room temperature gave high yields of the corresponding esters. These were identified by n.m.r. and mass spectroscopy together with elemental analysis. N.m.r. studies also suggest that bilirubin dimethyl ester is in the lactam rather than the lactim form.

Enzymes found in liver catalyse the formation of water-soluble derivatives of bilirubin (I) (Lathe, 1972). This conjugation process is necessary if bilirubin is to be excreted in bile and seems in most instances to be an esterification of the propionic acid groups of bilirubin with uronic acids or simple sugars such as D-glucose and D-xylose (Heirwegh *et al.*, 1970; Fevery *et al.*, 1971; Compernolle *et al.*, 1971). It is likely that most bilirubin in human bile is conjugated with β-D-glucopyranuronic acid (Billing *et al.*, 1957), but some appears to be excreted in conjugation with more complex oligosaccharides (Kuenzle, 1970*b*).

Precise analysis of the importance of the various conjugates is hindered by the difficulty of isolating pure specimens of conjugated bilirubin from bile. The available methods are lengthy, and the purity of the products is questionable, either because separation is incomplete, or because artifacts are formed during isolation (Ostrow & Murphy, 1970, and references therein). Synthetic studies are needed to provide samples of bilirubin conjugates of defined structure for comparison with material isolated from bile. No successful synthesis of a bilirubin conjugate has been reported to date: the material isolated by Thompson & Hofmann (1970) was grossly impure.

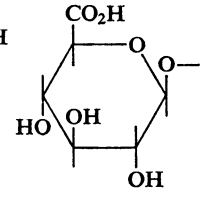
These considerations prompted us to begin a series of studies directed to achieving the synthesis of a series of sugar and uronic acid conjugates of bilirubin. Any chemical method for the synthesis of bilirubin conjugates must allow the stereospecific esterification of a particular hydroxyl group of a polyhydroxylated saccharide residue. Moreover, in view of the known instability of bilirubin in acid (McDonagh & Assisi, 1972) or alkali (Fog & Bugge-Asperheim, 1964; De Ewenson *et al.*, 1966), the esterification must take place under mild conditions and with the minimum use of protecting groups. The methods currently

* To whom reprint requests should be sent.

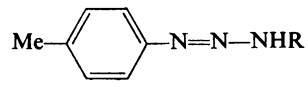


(I) R = H

(II) R =



(III) (a) R = Me, (b) R = Et, (c) R = iPr, (d) R = CH_2Ph



(IV) (a) R = Me, (b) R = Et, (c) R = *n*Pr, (d) R = iPr, (e) R = CH_2Ph

available for the synthesis of the dimethyl ester of bilirubin, such as those that use diazomethane (Fischer *et al.*, 1941), methanol–BF_3 (Cole *et al.*, 1967), or methanol–HCl (Nichol & Morell, 1969), are unsuitable as these procedures cannot be easily adapted for the acylation of specific hydroxyl groups in mono- or oligo-saccharides. 1-Alkyl-3-*p*-tolyltriazenes(IV) will esterify carboxylic acids rapidly and in high yield under mild conditions (White & Scherrer, 1961; White *et al.*, 1968). We here report the use of these compounds for the preparation of a number of hitherto unknown alkyl and aralkyl esters of bilirubin (III b, c, d).

Experimental

Materials

Bilirubin obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.) was purified by the method of Fog (1964) before use (Found: C, 67.9; H, 6.0; N, 9.2; $C_{33}H_{36}N_4O_6$ requires: C, 67.8; H, 6.2; N, 9.6%); it had a light-absorption maximum in chloroform at 454nm (ϵ 59400). That from Fluka A.G. (Buchs, Switzerland) and Sigma (London) Chemical Co., London S.W.6, U.K., was used without further purification. 1-Alkyl-3-*p*-tolyltriazenes were obtained from Willows Brook Laboratories Inc. (Waukesha, Wis., U.S.A.) and were used without further purification. ^1H n.m.r. spectra were recorded at 100MHz with a Varian HR-100 spectrometer. Mass spectra were recorded with an AEI MS9 instrument. Both mass and n.m.r. spectra were recorded at Physico-Chemical Measurements Unit, Harwell, U.K.

Methods

Preparation of bilirubin esters. Bilirubin dimethyl ester (IIIa). Bilirubin (I) (120mg, 0.2mmol) was suspended in chloroform (120ml) and 1-methyl-3-*p*-tolyltriazene (IVa) (150mg, 1.0mmol) was added. The mixture was stirred, in the dark, at room temperature for 12h. During this time, the reaction mixture darkened and the bilirubin was dissolved. Excess of triazene was decomposed by shaking the chloroform solution with dilute HCl (1M, 100ml). Traces of acid were removed from the chloroform solution by shaking with saturated aq. $NaHCO_3$ (100ml). Finally, the chloroform solution was shaken with water (2× 100ml) and dried (anhydrous Na_2SO_4). After filtration and removal of the solvent *in vacuo* at 40°C, the residue was applied to a column (45cm×1.5cm external diam.) of neutral alumina. Elution with chloroform–light petroleum (b.p. 60–80°C) (1:1, v/v) followed by pure chloroform removed most of the impurities. The bilirubin dimethyl ester was eluted with chloroform–methanol (20:1, v/v), and was finally purified by preparative t.l.c. on silica gel and by developing the chromatogram with benzene–ethanol (25:2, v/v) (Petryka & Watson, 1968). The orange band (R_F 0.7) was removed from the plate and the ester was eluted from the adsorbent with chloroform. Removal of the chloroform followed by crystallization of the residue from methanol gave bilirubin dimethyl ester (56.5mg, 46%), m.p. 198–200°C, $\lambda_{max.}$ in chloroform 400nm (ϵ 55600) (Found: C, 68.6; H, 7.1; N, 8.9. Calc. for $C_{35}H_{40}N_4O_6$: C, 68.6; H, 6.6; N, 9.2%).

Bilirubin diethyl ester (IIIb) was synthesized from bilirubin (120mg, 0.2mmol) and 1-ethyl-3-*p*-tolyltriazene (IVb) 325mg, 2.0mmol) as described above. Crystallization from hexane of the product obtained from t.l.c. gave *bilirubin diethyl ester* (13mg, 10%), m.p. 197–198°C, $\lambda_{max.}$ in chloroform 400nm (ϵ 55100) (Found: C, 69.4; H, 6.9; N, 8.3; $C_{37}H_{44}N_4O_6$ requires: C, 69.3; H, 6.9; N, 8.7%).

Bilirubin di-isopropyl ester (IIIc) was synthesized from bilirubin (120mg, 0.2mmol) and 1-isopropyl-3-*p*-tolyltriazene (IVd) (355mg, 2.0mmol) as described above. Crystallization from hexane of the product obtained from t.l.c. gave *bilirubin di-isopropyl ester* (20mg, 15%), m.p. 179–181°C, $\lambda_{max.}$ in chloroform 400nm (ϵ 54200) (Found: C, 69.6; H, 7.3; N, 8.5; $C_{39}H_{48}N_4O_6$ requires C, 70.0; H, 7.2; N, 8.4%).

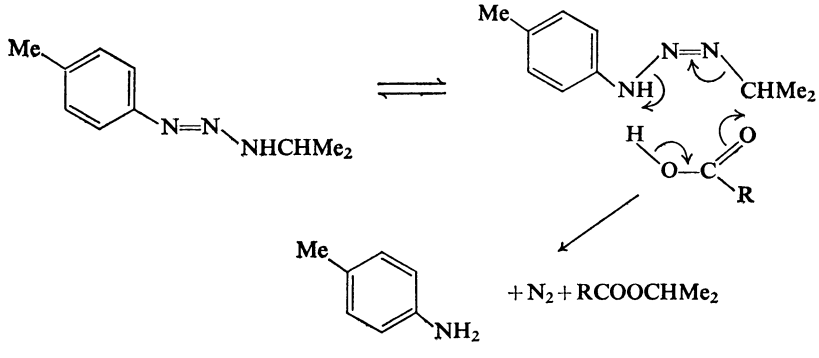
Bilirubin dibenzyl ester (IIId) was synthesized from bilirubin (120mg, 0.2mmol) and 1-benzyl-3-*p*-tolyltriazene (IVe) (450mg, 2.0mmol) as described above. The material obtained from the thin-layer chromatogram (23mg, 14%) had $\lambda_{max.}$ in chloroform 400nm. Despite repeated attempts this material could not be crystallized and an analytically pure sample was not obtained. The n.m.r. and mass spectra of the compound were consistent with the structure of the *bilirubin dibenzyl ester.*

Reaction between phenylacetic acid and 1-*n*-*propyl*-3-*p*-*tolyltriazene.* To phenylacetic acid (272mg, 2mmol) in chloroform (50ml) was added 1-*n*-propyl-3-*p*-tolyltriazene (IVc) (390mg, 2.2mmol) with stirring. Immediate evolution of N_2 was observed and the solution was left at room temperature for 30min. Excess of triazene was removed with HCl in the usual manner, the chloroform solution was dried (anhydrous Na_2SO_4) and evaporated to leave a yellow oil which was examined by n.m.r. spectroscopy without further purification. No signals corresponding to isopropyl groups could be detected in the n.m.r. spectrum of the oil.

Results and Discussion

1-Alkyl-3-*p*-tolyltriazenes react with bilirubin under mild conditions to give bilirubin alkyl esters. It is likely that attack by the carboxylic residues of bilirubin on the alkyl group of one tautomer of the triazene produces the ester, N_2 and *p*-toluidine (Scheme 1). The stereospecificity of the displacement reaction is unknown, but we believe that the decomposition of the alkyltriazenes in chloroform does not involve carbonium ions as intermediates. Examination by n.m.r. spectroscopy of the reaction product from phenylacetic acid and 1-*n*-propyl-3-*p*-tolyltriazene failed to reveal any signals due to isopropyl groups, indicating that the reaction had proceeded without the rearrangement of a hypothetical carbonium ion intermediate. The methyl, ethyl, isopropyl and benzyl esters of bilirubin have all been prepared by this method and have been identified by n.m.r., mass spectrometry and elemental analysis. Accurate mass values for the molecular ions of the esters (Table 1) confirm their molecular formulae and the low-resolution mass spectra confirm their proposed structures. For example, the mass spectrum of bilirubin di-isopropyl ester (Fig. 1) shows a parent ion at 668.3574 corresponding to $C_{39}H_{48}N_4O_6$. The fragmentation pattern in the low-resolution spectrum shows major peaks at *m/e* 341 and 328 corresponding to the molecule breaking into two fragments at the central methylene bridge. The ^1H n.m.r. spectrum of bilirubin di-isopropyl ester showed a doublet at 1.23 p.p.m. (6*H*), a septet centred at 5 p.p.m. (1*H*) and other signals from the bilirubin residue which were common to the n.m.r. spectra of all bilirubin esters examined (Fig. 2, Table 2).

N.m.r. spectroscopy confirms that rings A and D of bilirubin esters are in the lactam rather than the lactim form. Addition of traces of water to a solution of bilirubin dimethyl ester in either [$^2H_6$]dimethyl sulphoxide or deuterochloroform does not cause an alteration in the low field signals in the n.m.r. spectra. This suggests that these signals are due to N-*H* rather than O-*H* protons (Hutchinson *et al.*, 1971) and that the lactam tautomer predominates in these solvents. There are differences in the chemical shifts of the C-methyl protons of bilirubin dimethyl ester when the spectra are recorded of solutions of the ester in [$^2H_6$] dimethyl sulphoxide or deuterochloroform. We support the suggestion (Kuenzle, 1970*a*) that these differences are due to solvent effects rather than a change in the lactam/lactim ratio. Low-temperature n.m.r. spectroscopy might be expected to freeze an amido–imidol equilibrium and reveal signals due to both the lactam and lactim forms of bilirubin esters. However, on lowering the temperature of a solution of



Scheme 1. *Reaction of bilirubin with 1-alkyl-3-p-tolyltriazene under mild conditions*

For details see the text.

Table 1. *Accurate mass values for the parent ions in the mass spectra of bilirubin esters*

For details see the text.

Ester	Molecular formula	Required	Found
Dimethyl	$C_{35}H_{40}N_4O_6$	612.2948	612.2943
Diethyl	$C_{37}H_{44}N_4O_6$	640.3261	640.3271
Di-isopropyl	$C_{39}H_{48}N_4O_6$	668.3574	668.3582
Dibenzyl	$C_{47}H_{48}N_4O_6$	764.3574	764.3596

Table 2. *N.m.r. assignments for spectra of bilirubin esters in deuterochloroform*

Chemical shifts are given in p.p.m. relative to tetramethylsilane = 0. The shape, relative area and coupling constants for each signal are given in parentheses after their chemical shifts. The abbreviations used are: s, singlet; d, doublet; t, triplet; q, quartet; sp, septet; m, multiplet.

Dimethyl ester	Diethyl ester	Di-isopropyl ester	Dibenzyl ester	Assignment
		1.23 (d, 12*H*, $J = 7$Hz)		Methyl protons of isopropyl ester groups
	1.26 (t, 6*H*, $J = 7$Hz)			Methyl protons of ethyl ester groups
1.73 (s,) 1.96 (s,) 12*H* 2.08 (s,)	1.73 (s,) 1.97 (s,) 12*H* 2.80 (s,)	1.74 (s,) 1.96 (s,) 12*H* 2.09 (s,)	1.70 (s,) 1.96 (s,) 12*H* 2.05 (s,)	Methyl groups on C-2, C-7, C-13 and C-17
2.68 (A_2B_2 system, 8*H*)	2.66 (A_2B_2 system, 8*H*)	2.64 (A_2B_2 system, 8*H*)	2.68 (A_2B_2 system, 8*H*)	Methylene protons of propionic ester side chains on C-8 and C-12
3.69 (s, 6*H*)	—	—	—	Methyl protons of methyl ester groups
	4.15* (q, $J = 7$Hz)			Methylene protons of ethyl ester groups
4.16 (s-broad, 2*H*)	*	4.18 (s-broad, 2*H*)	4.14 (s-broad, 2*H*)	Methylene protons on C-10
4.72–6.68 (m, 8*H*)	4.72–6.70 (m, 8*H*)	4.72–6.70 (m, 8*H*)	4.74–6.68 (m)†	Vinyl groups on C-3 and C-18 and methine protons on C-5 and C-15
		5.02 (sp, 2*H*, $J = 7$Hz)		Methine protons of isopropyl ester groups
			5.11 (s)†	Methylene proton of benzyl ester groups
			7.30 (s, 10*H*)	Phenyl ring protons of benzyl ester groups
10.10–11.18 (m-broad, 4*H*)	10.10–11.16 (m-broad, 4*H*)	10.08–11.18 (m-broad, 4*H*)	10.07–11.14 (m-broad, 4*H*)	Protons on ring nitrogen atoms

* The quartet and methylene singlet overlap; the total integral corresponds to 6*H*.
† Total integral corresponds to 12*H*.

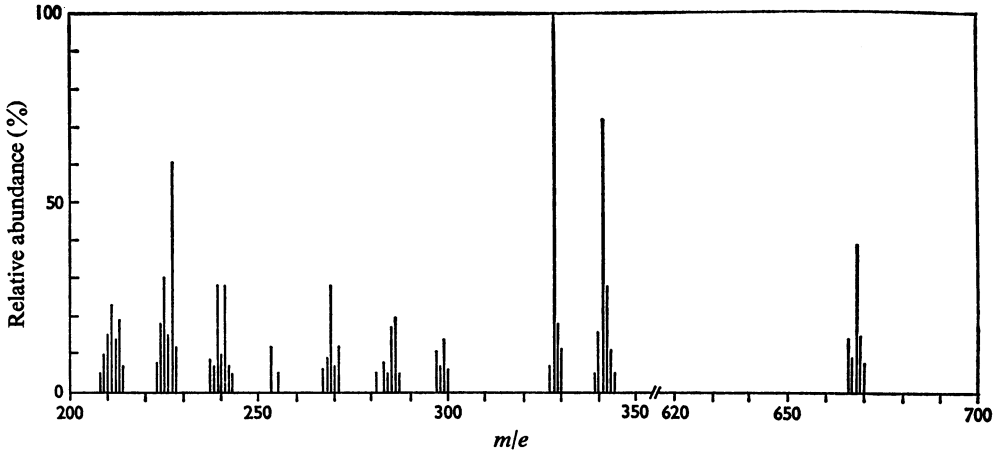


Fig. 1. *Low-resolution mass spectrum of bilirubin di-isopropyl ester*

The peak at *m/e* 668 corresponds to the parent ion $C_{39}H_{48}N_4O_6$; cleavage at the central methylene bridge occurs on electron impact giving a peak at *m/e* 341 and the base peak at *m/e* 328. The fragmentation pattern below *m/e* 200 is very similar to that for bilirubin (Jackson *et al.*, 1967).

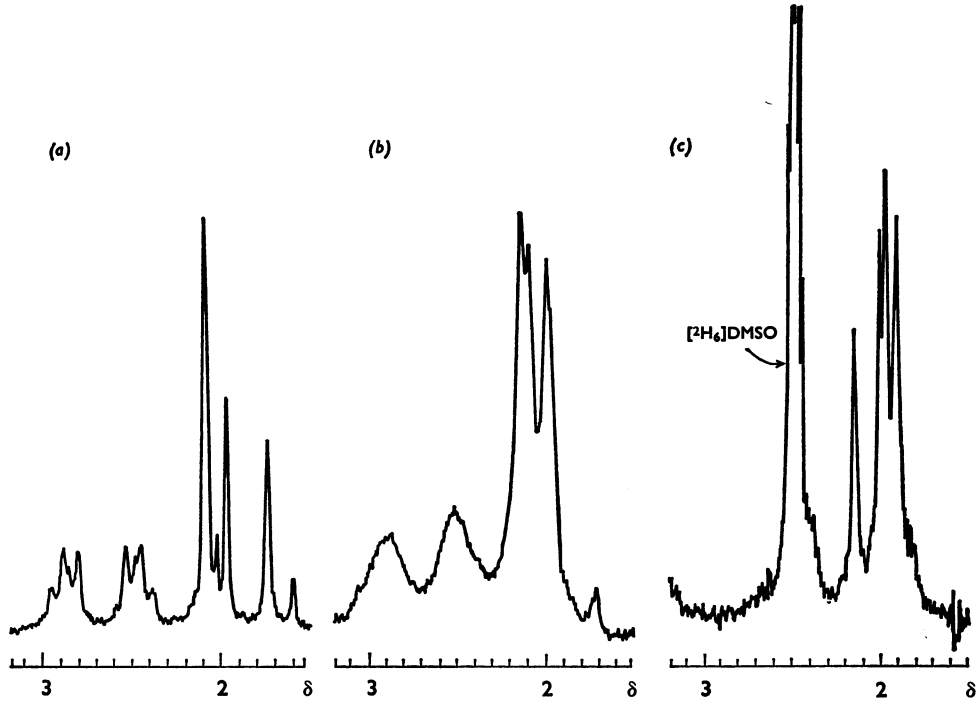


Fig. 2. *N.m.r. spectra of the C-methyl protons of bilirubin dimethyl ester in (a) deuterochloroform at ambient temperature, (b) deuterochloroform at −60°C and (c) [$^2H_6$]dimethyl sulphoxide ([$^2H_6$]DMSO) at ambient temperature*

Chemical shifts are given in p.p.m. relative to tetramethylsilane = 0 (internal standard). Assignments are given in the text.

bilirubin dimethyl ester in deuterochloroform no new signals appeared, the signal at 1.72p.p.m. moved to lower field and the signal at 2.08p.p.m. (probably due to pyrrole C-methyl protons) split into a barely resolved doublet. This behaviour is consistent with free rotation about the central methylene bridge being decreased as the temperature is lowered and the two halves of the molecule becoming non-equivalent. The previously reported n.m.r. spectrum of bilirubin dimethyl ester in deuterochloroform shows several small high-field peaks which have been ascribed (Kuenzle, 1970*a*) to the C-methyl protons of several different molecular species present in the ester. We have found that when bilirubin dimethyl ester was prepared from different commercial samples of bilirubin, the proportions of the sizes of these small peaks varied and were independent of concentration. Thus it is unlikely that these small peaks arise from lactam–lactim tautomerism of the ester in deuterochloroform and we believe that they are probably due to small amounts of bilirubin-like impurities present in the different commercial samples of bilirubin.

Thus this method appears to be suitable for the synthesis of conjugates of bilirubin if triazenes derived from amino sugars are used. Such amino sugars are known (Hodge & Moy, 1963), so it will be of interest to study this synthetic method further.

We thank the Medical Research Council for financial support.

References

Billing, B. H., Cole, P. G. & Lathe, G. H. (1957) *Biochem. J.* **65**, 774–784

Cole, W. J., Chapman, D. J. & Siegelman, H. W. (1967) *J. Amer. Chem. Soc.* **89**, 3643–3645

Compernolle, F., Van Hees, G. P., Fevery, J. & Heirwegh, K. P. M. (1971) *Biochem. J.* **125**, 811–819

De Ewenson, I. W., Gianturco, F. A. & Gramaccioni, P. (1966) *Experientia* **22**, 14–15

Fevery, J., Van Hees, G. P., Leroy, P., Compernolle, F. & Heirwegh, K. P. M. (1971) *Biochem. J.* **125**, 803–810

Fischer, H., Plieninger, M. & Weissbarth, O. (1941) *Hoppe-Seyler's Z. Physiol. Chem.* **268**, 197–226

Fog, J. (1964) *Scand. J. Clin. Lab. Invest.* **16**, 49–54

Fog, J. & Bugge-Asperheim, B. (1964) *Nature (London)* **203**, 756–766

Heirwegh, K. P. M., Van Hees, G. P., Compernolle, F. & Fevery, J. (1970) *Biochem. J.* **120**, 17P

Hodge, J. E. & Moy, B. F. (1963) *J. Org. Chem.* **28**, 2784–2789

Hutchinson, D. W., Johnson, B. & Knell, A. J. (1971) *Biochem. J.* **123**, 483–484

Jackson, A. H., Kenner, G. W., Budzikiewicz, H., Djerassi, C. & Wilson, J. M. (1967) *Tetrahedron* **23**, 603–632

Kuenzle, C. C. (1970*a*) *Biochem. J.* **119**, 395–410

Kuenzle, C. C. (1970*b*) *Biochem. J.* **119**, 411–435

Lathe, G. H. (1972) *Essays Biochem.* **8**, 107–148

McDonagh, A. F. & Assisi, F. (1972) *J. Chem. Soc. D* 117–118

Nichol, A. W. & Morell, D. B. (1969) *Biochim. Biophys. Acta* **177**, 599–609

Ostrow, J. D. & Murphy, N. H. (1970) *Biochem. J.* **120**, 311–327

Petryka, Z. J. & Watson, C. J. (1968) *J. Chromatogr.* **37**, 76–82

Thompson, R. P. H. & Hofmann, A. F. (1970) *Gastroenterology* **60**, 202

White, E. H. & Scherrer, H. (1961) *Tetrahedron Lett.* 758–762

White, E. H., Baum, A. A. & Eitel, D. E. (1968) *Org. Syn.* **48**, 102–105