SYNTHESIS OF BILIVERDIN AND BILIRUBIN GLUCURONIDES

Frans COMPERNOLLE*

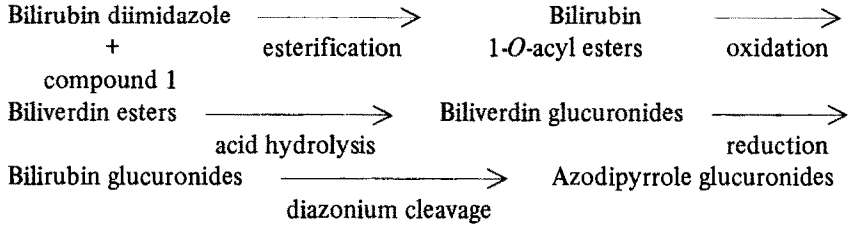
Laboratory of Hepatology and Laboratory of Organic Chemistry, University of Leuven, B-3000 Leuven, Belgium

Received 25 February 1980

1. Introduction

Bilirubin, the major degradation product of heme, is excreted by mammalian liver cells as mono- and di-β-glycosidic esters with glucuronic acid (fig.1), glucose and xylose [1–5]. None of these conjugates has been isolated in pure form, while previous attempts at their chemical synthesis have failed [6–8]. Esterification of unprotected glucuronic acid with bilirubin diimidazole yielded a mixture of non C-1-glururonides [6]. Deprotection of biliverdin or bilirubin 1-*O*-acyl allyl 2,3,4-tri-*O*-allyl-D-glucopyranuronates resulted in degradation of the tetrapyrrole chromophores [8].

The present report describes the first successful synthesis of biliverdin and bilirubin glucuronides. Compound 1, an acetal derivative of glucuronic acid [9], and bilirubin diimidazole [6,8] served as key starting materials (fig.2) for the reaction sequence outlined below. The mild acidic conditions used for removal of the acetal groups prevent base-catalyzed migration of the 1-*O*-acyl group to positions 2,3 and 4 of glucuronic acid [3,4], but necessitate oxidation of intermediate bilirubin esters to biliverdins since bilirubins disproportionate readily into a mixture of the isomers-IXα, -XIIIα and -IIIα (A-CH_2-B, A-CH_2-A and B-CH_2-B, fig.1) [10].



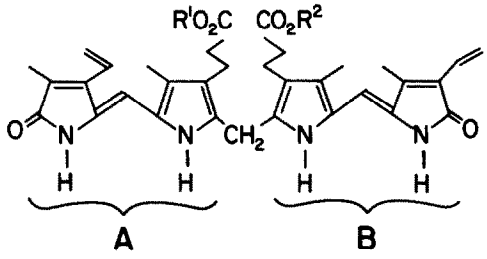


Fig.1. Bilirubin, $R^1 = R^2 = H$. Bilirubin diglucuronide, $R^1 = R^2 = \beta$-D-glucopyranuronic acid. Bilirubin monoglucuronide, $R^1 = \beta$-D-glucopyranuronic acid, $R^2 = H$ or vice versa.

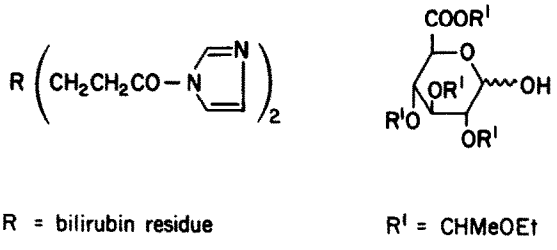


Fig.2. Bilirubin diimidazole, with propionic acid side chains activated, and compound 1, an acetal-protected derivative of glucuronic acid.

2. Methods and results

2.1. *Synthesis of biliverdin glucuronides*

2.2.1. Procedure

A solution of 50 mg compound 1 [9] and bilirubin diimidazole [6,8], prepared from 10 mg of bilirubin, in 1 ml ethanol-free chloroform was evaporated and the residue was heated at 75°C for 50 min (dark, vacuum), cooled and dissolved in 10 ml dichloromethane. The solution was shaken with 10 mg DDQ (freshly dissolved in 10 ml water) for 2–3 min, washed with water (5 × 10 ml) and evaporated. The residue was hydrolyzed (22°C, 30 min) with 1 ml acetic acid and 0.25 ml 0.1 M HCl; reagents were evaporated at 22°C in vacuo. The major reaction products, biliverdin mono-α-, mono-β- and di-αβ-glucuronides, were isolated by thin-layer chromatography (for R_F-values see table 1) on silica gel with solvent system A ($CHCl_3$–MeOH–H_2O–HOAc 100:50:10:1; elution with MeOH–HOAc 3:1). Yields mentioned in table 1 were calculated from $A_{376\,(max)}$ and $A_{666\,(max)}$, assuming ϵ = 50 800 and 14 400 as for biliverdin [10].

2.1.2. Characterization

A reference mixture of biliverdin mono-β- and di-β-glucuronides was prepared by oxidation of the natural bilirubin β-glucuronides with DDQ. The slower moving β-anomer of synthetic biliverdin monoglucuronide and the synthetic biliverdin di-αβ-glucuronide co-chromatographed with the reference biliverdins. Dissolution of the faster moving α-anomer of biliverdin monoglucuronide in water (pH 6–7) or omission of acetic acid from TLC solvents gave rise to migration of the α-1-*O*-acyl group and formation of biliverdin 2-*O*-acylglucuronide. This compound moved as a third band (R_F 0.30) below biliverdin mono-α and β-glucuronides and yielded the known azodipyrrole 2-*O*-acylglucuronide methyl ester [3] after $NaCNBH_3$ reduction, diazonium cleavage and treatment with diazomethane (for procedures see below under Synthesis of bilirubin glucuronides).

^{1}H NMR spectra of 1:1 anomeric mixtures of biliverdin mono- and diglucuronides (obtained by large-scale TLC separation) were recorded in [^{2}H]DMSO-[^{2}H]HOAc (5:1) solutions with a Varian XL 100 spectrometer. Signals for the anomeric protons were observed as doublets at δ 5.40 (β-form, $J_{1,2}$ = 7 Hz) and 5.98 (α-form $J_{1,2}$ = 2.5 Hz). Other signals were due to the ring protons of the sugar (δ 3.1–4.1) and to various protons of the biliverdin moiety: methyl groups (δ 2.11 and 2.17), propionic side chains (δ 2.95) and C-5 and C-15 methine protons (δ 6.12).

Hydrolysis with β-glucuronidase was performed as described for azodipyrrole glucuronides [11]. After incubation (pH 5.2, 37°C, 1–2 h), biliverdins were extracted by addition of glycine-HCl buffer (pH 1.8) and butanol, and assessed by TLC using solvent system A (table 1). Biliverdin mono-β-glucuronide was hydrolyzed completely, whereas the mono-α-glucuronide resisted the action of the enzyme. Treatment of biliverdin di-αβ-glucuronide yielded biliverdin, biliverdin mono-α-glucuronide and biliverdin di-α-glucuronide (~1:2:1 ratio, table 1).

Alkaline hydrolysis of biliverdin glucuronides was carried out in 0.1 M NaOH (dark, argon). After 1 h the solutions were acidified with glycine-HCl buffer (pH 1.8) and extracted with butanol. The extract was evaporated in vacuo and the residue dissolved in acetic acid. $NaCNBH_3$ was added and bilirubin extracted by addition of chloroform and water. The IIIα/IXα/XIIIα isomer composition of the isolated bilirubin was deter-

Table 1
Properties of biliverdin and bilirubin mono- and di-1-*O*-acyl-α and β-D-glucopyranuronic acids

Biliverdin glucuronides isolated by TLC	BVMGα	BVMGβ	BVDG
Yields (based on bilirubin)	6%	6%	18%
R_F-values (solvent system A)	0.36	0.33	0.13
R_F-values of bilirubin reduction products	0.50	0.46	0.12
Hydrolysis products with β-glucuronidase	BVMGα	BV	BV 25% BVMGα 54% BVDG 21%

Abbreviations: BV, biliverdin; BVMG, biliverdin monoglucuronide; BVDG, biliverdin diglucuronide

mined by TLC ($CHCl_3$–HOAc 99:1) [10] and was similar (2.5:91:6.5) to that of the starting bilirubin (Merck, 2.5:92:5.5).

2.2. *Synthesis of bilirubin glucuronides*

Purified biliverdin mono-α-, and mono-β- and di-αβ-glucuronides (0.1–1 mg) in HOAc–iPrOH (1:9) were shaken with $NaCNBH_3$ (1–3 mg) for 1–3 min. When kept in the presence of this reagent, the resulting solutions of bilirubin glucuronides can be stored (0°C, dark) for prolonged periods of time without deterioration, i.e. oxidation or dipyrrole exchange as shown by TLC of bilirubin monoglucuronides. Bilirubin glucuronides were isolated by TLC as the only reaction products (solvent system A, elution with MeOH or MeOH–HOAc 9:1). Separation of the anomeric monoglucuronides occurred on TLC (table 1) and the lower moving β-anomer co-chromatographed with the natural bilirubin monoglucuronide. Identical R_F-values were observed also for the diglucuronides.

The unstable bilirubin tetrapyrroles were characterized by cleavage to dipyrrolic azopigments using an ethyl anthranilate diazonium reagent [12]. Treatment of the α- and β-monoglucuronide yielded an equimolar mixture of free azodipyrrole acid and azodipyrrole glucuronide. Only azodipyrrole αβ-glucuronide was formed from bilirubin di-αβ-glucuronide. The azodipyrrole glucuronides were isolated by TLC using solvent system A (elution with 2-pentanone and glycine buffer, pH 2.7) and esterified by brief treatment with diazomethane (1–3 s). The resulting anomeric methyl esters were separated by TLC with $CHCl_3$–MeOH (9:1, 2 developments). Whereas the lower moving anomer was identical with the azopigment derived from natural bilirubin β-glucuronides, the α-anomer differed in R_F-value from the previously [3,4] characterized 2-, 3- and 4-*O*-acyl glucuronide methyl esters. Final confirmation of structure was provided by the mass spectra of trimethylsilyl derivatives of azodipyrrole glucuronide methyl esters, showing the expected molecular ions at m/e 796 and 868 (two and three silyl groups) [3,13].

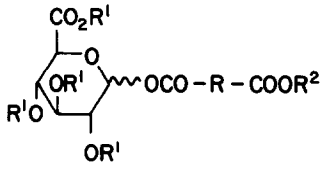


Fig.3. Mono-esters (R^2 = H) and diesters (R^2 = glucuronide moiety) of bilirubin and biliverdin. R = tetrapyrrole residue; R^1 = -CHMeOEt for acetal protected or H for deprotected glucuronides.

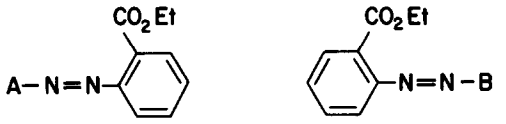


Fig.4. Ethyl anthranilate dipyrrolic azopigments. A and B are isomeric dipyrrole halves of bilirubin or bilirubin glucuronides (fig.1).

3. Discussion

The synthetic pathway is outlined in section 1. Compound 1, a mixture of diastereoisomers (fig.2), was prepared [9] from methyl tetra-*O*-acetyl-α or β-D-glucopyranuronate via the α-1-bromo and β-1-*O*-benzyl derivatives, followed by alkaline hydrolysis, acetal formation and hydrogenolysis. Esterification of compound 1 with bilirubin diimidazole yielded both mono- and di-1-*O*-acyl esters of bilirubin (fig.3). To avoid disproportionation during subsequent acid hydrolysis of acetal groups, the bilirubin esters were oxidized to the corresponding biliverdin esters by treatment with 2,3-dichloro-5,6-di-cyano-1,4-benzoquinone (DDQ). Mild acid hydrolysis yielded mono- and mainly di-1-*O*-acyl-D-glucopyranuronic acid derivatives of biliverdin, present as 1:1 anomeric mixtures (table 1). Bilirubin mono-α, mono-β- and di-αβ-glucuronides were obtained by reduction of the corresponding biliverdins and characterized as dipyrrolic azopigments (fig.4) and their methyl ester and silyl ether derivatives (fig.5).

The present synthesis confirms the glucuronide structure recently [3,5] proposed for the major bili-

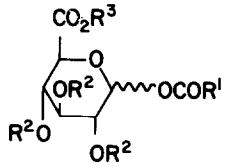


Fig.5. Azodipyrrole glucuronide (R^1 = azodipyrrole residue; R^2 = R^3 = H), methyl ester (R^2 = H, R^3 = Me) and methyl ester silyl ether (R^2 = Me_3Si, R^3 = Me).

rubin conjugates of human and rat bile. Appropriate modifications will provide access to various labelled derivatives and to other carbohydrate conjugates, which are needed for the study of hepatic transport and for elucidation of the enzymic mechanism of bilirubin conjugation. The synthetic bilirubin glucuronides can also serve as reference materials for analysis of conjugated bilirubin. Further efforts are now directed towards the specific synthesis of bilirubin β-glucuronides.

References

[1] Compernolle, F., Van Hees, G. P., Fevery, J. and Heirwegh, K. P. M. (1971) Biochem. J. 125, 811–819.
[2] Gordon, E. R., Chan, T.-H., Samodai, K. and Goresky, C. A. (1977) Biochem. J. 167, 1–8.
[3] Compernolle, F., Van Hees, G. P., Blanckaert, N. and Heirwegh, K. P. M. (1978) Biochem. J. 171, 185–201.
[4] Blanckaert, N., Compernolle, F., Leroy, P., Van Houtte, R., Fevery, J. and Heirwegh, K. P. M. (1978) Biochem. J. 171, 203–214.
[5] Compernolle, F. (1978) Biochem. 175, 1095–1101.
[6] Thompson, R. P. H. and Hofmann, A. H. (1976) Biochim. Biophys. Acta 451, 267–277.
[7] Hancock, F. E., Hutchinson, D. W. and Knell, A. J. (1976) Biochem. Soc. Trans. 4, 303–304.
[8] Compernolle, F. (1980) Carbohydr. Res. in press.
[9] Compernolle, F. (1980) Carbohydr. Res. in press.
[10] McDonagh, A. F. (1979) in: The Porphyrins (Dolphin, D. ed), vol. 6, pp. 293–491, Academic Press, New York.
[11] Blanckaert, N., Fevery, J., Heirwegh, K. P. M. and Compernolle, F. (1977) Biochem. J. 164, 237–249.
[12] Van Roy, F. P. and Heirwegh, K. P. M. (1968) Biochem. J. 107, 507–518.
[13] Compernolle, F., Van Hees, G. P. and Heirwegh, K. P. M. (1978) Biomed. Mass Spectrom. 5, 453–459.