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IN VIVO STUDIES OF HEPATOBILIARY EXCRETION OF BILIRUBINS

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Abstract: Hepatic metabolism of synthetic biliverdins 18-ethyl-1,19,21,24-tetrahydro-2,8,13,17-tetramethyl - 1,19-dioxobilin-3,7,12-tripropionic acid (**6**) and coprobiliverdin II β (**7**) – substrates in vitro of biliverdin reductase – showed that increasing hydrophilicity of bilirubins leads to their biliary excretion without conjugation.
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Bilirubin IX α, an intermediate in heme IX catabolism in mammals, is a hydrophobic pigment and requires conjugation, which provides water solubility to the aglycon thus facilitating its excretion. [1] Conjugation – mainly with glucuronic acid – to give bilirubin IX α monoglucuronide and bilirubin IX α diglucuronide, takes place in the liver and UDP-glucuronosyltransferase is the enzyme capable of this process. [2-4]

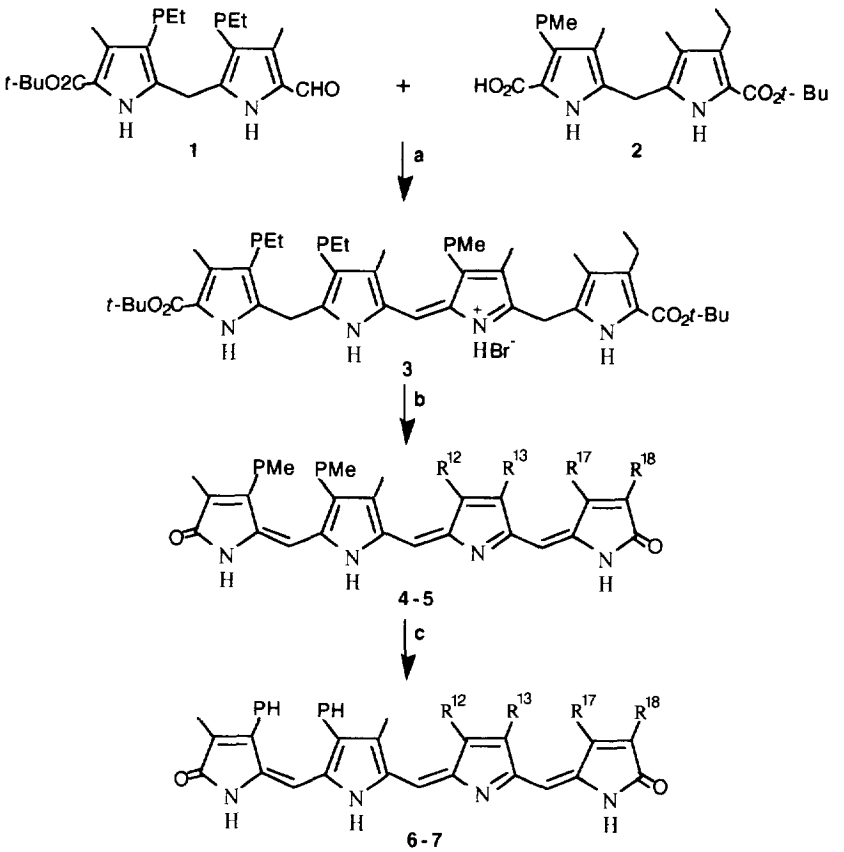
It has been proposed that bilirubin does not need to have two carboxyl groups to bind to the active site of UDP-sugar-transferases.[5,6] On the other hand, intramolecular hydrogen bonds between the COOH/COO $^-$ and NHCO/NH of bilirubin IX α seem to be important for bilirubin conjugation. [7]

In order to study UDP-glucuronosyltransferase specificity two synthetic biliverdins, efficiently reduced in vitro to bilirubins by biliverdin reductase, were prepared. [8,9] Polarity and the possibility of intramolecular hydrogen bonding of bilirubins were specially considered for the synthetic design.

A biliverdin replaced with three propionic acid chains on C-3, C-7, and C-12 and a biliverdin substituted by four propionic acid chains on C-3, C-7, C-13, and C-17, were injected into the Wistar rat femoral vein and the biliary excretion after their metabolism was analyzed.

Chemistry

Biliverdins **4** and **5** were obtained as methyl esters by total synthesis in a fashion similar to that of an earlier work.[10] Condensation of dipyrrylmethanes **1**[11] and **2**[12] gave the 1,19-di-*t*-butoxycarbonyl-b-bilene hydrobromide **3**. The latter was hydrolyzed and decarboxylated in trifluoracetic acid and then carefully oxidized with bromine[10], followed by an alkaline workup. [10-13] Transesterification with sulphuric acid in methanol afforded biliverdins **4** and **5**[14,15] which were then saponified to the free acids by dissolution in a mixture of 1 vol of tetrahydrofuran, 1 vol of methanol, and 2 vol of 2 M NaOH. The solution was kept at 37 °C during 3.5 h, under nitrogen in the dark. It was then neutralized with glacial acetic acid and the biliverdins were extracted into chloroform, washed with water, dried over Na_2SO_4 and evaporated in vacuo at 35 °C to dryness.[15,16] (Scheme 1)



Reagents: (a) dry CH_2Cl_2; PTSA, dry CH_3OH: 20 °C, 14 h under N_2. (b) i. TFA, Br_2: 5 °C, 20 min under N_2; $NaCO_3H$. ii. dry CH_3OH, 5% H_2SO_4 (v/v): 5 °C, 12 h. (c) i. THF, CH_3OH, 2 M NaOH (1:1:2, v/v): 37 °C, 3.5 h under N_2. ii. glacial CH_3CO_2H.
4. R^{12}=PMe; R^{13}=R^{17}=Me; R^{18}=Et. **5**. R^{13}=R^{17}=PMe; R^{12}=R^{18}=Me
6. R^{12}=PH; R^{13}=R^{17}=Me; R^{18}=Et. **7**. R^{13}=R^{17}=PH; R^{12}=R^{18}=Me
PEt=$CH_2CH_2CO_2C_2H_5$; PMe= $CH_2CH_2CO_2CH_3$; PH=$CH_2CH_2CO_2H$; Me=CH_3; Et=C_2H_5

Scheme 1. Synthesis of biliverdins

Biological Assays

Male Wistar rats weighing 300-400 g were kept under ether anesthesia and the common bile duct was cannulated with 10 cm of PE-tubing and protected from the light with an aluminium-foil. The left femoral vein was also cannulated (PE-10 tubing, 10 cm) and the biliverdins **4** and **5** (0.9 x 10^{-7} mol) dissolved in 0.05 mL of 0.1 M NaOH and 1 mL of albumin solution (10% bovine albumin in 0.15 M saline solution w/v) were injected into the animal via the femoral vein for about 1 min with a 1 mL syringe. Continuous infusion (1 mL/h) of 0.15 M saline solution containing 5% glucose (w/v) was started after biliverdin injection and maintained for the duration of the experiment.[17] Room temperature was 20 °C throughout the length of the experiment and rat body temperature

was kept up by means of infrared lamps. The bile was collected in tubes placed on ice and protected from the light during common bile duct cannulation (t_0) the same as bile obtained at 30, 60, 90, 120, and 150 min after femoral vein injection of the biliverdin.

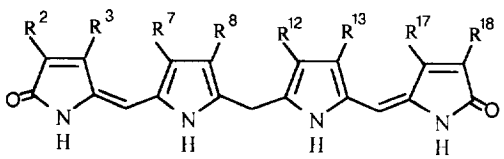
Extraction and separation of bilirubins from the bile were performed as described elsewhere[18] with some modifications.[19] The amount of bilirubin (Table 1) was determined by measuring the absorbance between 390 and 430 nm (ε = 43,526 M^{-1} cm^{-1} for bilirubin **8** and ε = 23,645 M^{-1} cm^{-1} for bilirubin **9**).[20] Bilirubin IX α conjugates were estimated by measuring the absorbance between 444 and 453 nm, ε = 60,000 M^{-1} cm^{-1}.

Identification of bilirubins **8** and **9** and bilirubin IX α glucuronides was performed by transformation into their azopigments using an established procedure,[18,19,21] after pooling the bilirubins of six assays.

Azo derivatives of the bile pigments were also prepared from whole bile. A mixture of bile sample (diluted 30-fold with water) (1 mL), 1 mL of citrate/phosphate buffer, pH 6.0 and 1 mL of diazo reagent was kept at 0 °C for 45 min in the dark and 1 mL of ascorbic acid solution (10 mg/mL) in glycine/HCl buffer, pH 2.0 was added. The azopigments were extracted without delay, after pooling twenty assays, with cold butyl acetate, washed with cold water and evaporated in vacuo at room temperature. Azopigments were performed with the bile samples at t_0, 30, 60, 90, 120, and 150 min.

In order to confirm that the system used for bilirubin extraction is quantitative and no conjugates of **8** and **9** remain in the aqueous phase, azopigments obtained from whole bile and from individual pigments were compared by TLC as described elsewhere.[18,19,21]

Synthetic bilirubins **8** and **9**, obtained by the chemical reduction of biliverdins **6** and **7** with sodium borohydride,[20] were also transformed into their azopigments and used as reference compounds in the TLC described above. The azopigments were then transformed into their methyl esters with diazomethane, compared by TLC,[18,19,21] and submitted to MS. Bilirubins **8** and **9** were also identified by FABMS.



8. $R^2=R^8=R^{13}=R^{17}=CH_3$; $R^{18}=C_2H_5$; $R^3=R^7=R^{12}=CH_2CH_2CO_2H$

9. $R^2=R^8=R^{12}=R^{18}=CH_3$; $R^3=R^7=R^{13}=R^{17}=CH_2CH_2CO_2H$

Scheme 2. Structure of bilirubins

Results and Discussion

In vivo experiments (Table 1) show that biliverdins **6** and **7** were efficiently metabolized into bilirubins **8** and **9** (Scheme 2) and excreted in the bile of male Wistar rats. After injection of biliverdins no significant variations in the bile flow and volume were observed. Bile fluid volumes, measured every 30 min, were 0.35 ± 0.04 mL.

Bilirubin **8** began to be excreted in the bile fluid and reached a maximum at 30 min injection of biliverdin **6** into the femoral vein, and then decreased at t_{60}, t_{90}, t_{120} and t_{150}. A similar excretion pattern was observed after biliverdin **7** was injected into the said vein.

The composition of bilirubin IX α conjugates present in the bile showed that bilirubin IX α monoglucuronide is the larger fraction at t_0, t_{30}, t_{60}, t_{90}, t_{120} and t_{150}.

Table 1. Separation by TLC of the components extracted from the bile fluids of cannulated vein rats injected with biliverdins. Bilirubins were extracted from the bile collected during common bile duct cannulation (t_0) and at 30, 60, 90, 120, and 150 min after biliverdins were injected into the femoral vein.

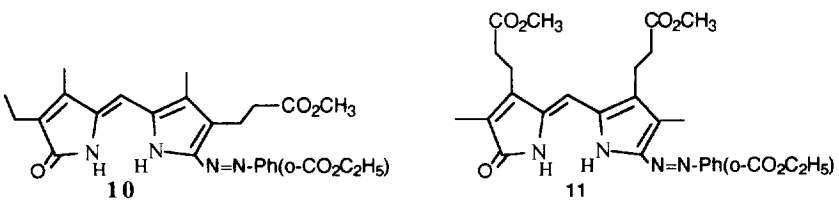
Biliverdin injected	Component	Concentration of bilirubin expressed in nmol/mL of bile					
		t_0	t_{30}	t_{60}	t_{90}	t_{120}	t_{150}
BV 6	BR IX α MG	15.35 ± 1.38	23.50 ± 1.40	24.63 ± 1.85	19.03 ± 0.75	15.20 ± 0.97	15.49 ± 1.17
	BR IX α DG	10.44 ± 0.94	14.55 ± 0.88	11.17 ± 0.69	8.62 ± 0.44	7.38 ± 0.53	8.35 ± 0.48
	BR 8	-	32.33 ± 1.80	19.84 ± 1.84	9.94 ± 0.75	6.39 ± 0.40	5.53 ± 0.53
	BV 6	-	1.76 ± 0.20	1.67 ± 0.13	1.30 ± 0.11	-	-
BV 7	BR IX α MG	16.13 ± 1.32	22.42 ± 1.01	16.02 ± 0.95	15.69 ± 0.17	14,53 ± 0.78	12.75 ± 1.22
	BR IX α DG	8.75 ± 0.60	10.04 ± 1.33	9.18 ± 1.20	7.50 ± 0.58	5.10 ± 0.47	5.30 ± 0.53
	BR 9	-	17.20 ± 1.48	14.12 ± 0.89	11.25 ± 0.70	8.25 ± 0.80	6.40 ± 0.90
	BV 7	-	-	-	-	-	-

BV: biliverdin; BR: bilirubin; MG: monoglucuronide; DG: diglucuronide.
Data represent mean ± SE for six rats.

FABMS of excreted bilirubins **8** and **9** (Table 2) and MS of azodipyrrole derivatives obtained from these bilirubins (Scheme 3) confirmed that bilirubins **8** and **9** were excreted in the bile without conjugation. Thus, bilirubin **8** afforded azopigments **10** MS (M^+= 478) and **11** MS (M^+= 536) whereas excreted bilirubin **9** gave azopigment **11** MS (M^+= 536).

The difference between the extinction coefficient of bilirubins **8** and **9** and that of bilirubin IX α can be taken as an indicator that bilirubin **8** has a folded conformation while bilirubin **9** has a porphyrin-like conformation.[22]

By means of ^{1}H NMR analysis of synthetic bilirubins **8** and **9**,[19] we found that N-H chemical shifts of pyrrole were at 9.2-7.7 for bilirubin **8** and at 9.45 for bilirubin **9**, and at 10.6-8.05 and 10.8 for lactam N-H signals thus showing that bilirubin **8** has intramolecular hydrogen bonding.[23]



Scheme 3. Structure of the azodipyrroles obtained from bilirubins **8** and **9**.

Bilirubin IX α – known to form intramolecular hydrogen bonding – is excreted in the bile conjugated[24] at least on one of its propionic chains.

Bilirubin **8** has both internally hydrogen bonded and exposed carboxyl groups while bilirubin **9** contains only exposed carboxyl groups. Exposed carboxyl groups make bilirubins **8** and **9** more polar than bilirubin IX α allowing their excretion in the bile without conjugation.

Our results indicate that the increasing hydrophilicity of the substrate leads to the excretion of unconjugated bilirubins, even when the substrate is able to form intramolecular hydrogen bonding as the natural substrate of UDP-glucuronosyltransferase bilirubin IX α.

Table 2. Spectrum data of bilirubins

BR	R_f[a]	FABMS[b], *m/z*	^{1}H NMR[c] (300 MHz, $CDCl_3$/TMS), δ ppm	
			Lactam	Pyrrole
8	0.80	784 $(M-H+m\text{-}NBA)^+$	10.60 - 8.05	9.20 -7.70
9	0.23	677 $(M+H)^+$	10.80	9.45

[a]Silica gel plates, 0.25 mm thickness, developed with $CHCl_3/CH_3OH/H_2O$, 48:28:6, v/v).

[b]m-nitrobenzyl alcohol was used as a matrix.

[c]Data of synthesis-obtained bilirubins **8** - **9**.[20]

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14. Biliverdins **4** and **5** were separated by TLC silica gel column. Elution with chloroform-10% acetone gave **4** (*Rf* 0.40) mp: 180 °C that was crystallized from methylene chloride-hexane (33% yield, dark blue

crystals). MS *m/z* 672 (M^+, 91%), 673 (M^++1, 100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.69 (s, 1H, HC(10)), 5.99 and 5.87 (2xs, 2H, HC(5, 15)), 3.70, 3.67 and 3.66 (3xs, 9H, $H_3C(3^5, 7^5, 12^5)$), 2.87 (m, 6H, $H_2C(3^1, 7^1, 12^1)$), 2.64, 2.52 and 2.47 (3xt, 6H, $H_2C(3^2, 7^2, 12^2)$), 2.32 (q, $^3J_{HH}$ = 7.5, 2H, $H_2C(18^1)$), 2.19 (s, 3H, $H_3C(8^1)$), 2.09 (s, 3H, $H_3C(13^1)$), 2.06 (s, 3H, $H_3C(17^1)$), 1.83(s, 3H, $H_3C(2^1)$), 1.06 (t, $^3J_{HH}$ = 7.5, 3H, $H_3C(18^2)$). Uv-vis (CH_3OH) λ_{max} 360 nm (48,063), 630 nm (13,786).

5 (*Rf* 0.20) was crystallized from methylene chloride-hexane, mp: 115 °C (lit[10] 115 °C) gave deep blue crystals (7.2% yield). MS *m/z* 730 (M^+, 20%), 360 (100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.71 (s, 1H, HC(10)), 6.01 (s, 2H, HC(5, 15)), 3.71 and 3.66 (2xs, 12H, $H_3C(3^5, 7^5, 13^5, 17^5)$), 2.80 (m, 8H, $H_2C(3^1, 7^1, 13^1, 17^1)$), 2.64 (t, $^3J_{HH}$ = 7.5, 4H, $H_2C(7^2, 13^2)$), 2.50 (t, $^3J_{HH}$ = 7.5, 4H, $H_2C(3^2, 18^2)$), 2.19 (s, 6H, $H_3C(8^1, 12^1)$), 1.82 (s, 6H, $H_3C(2^1, 18^1)$). Uv-vis (CH_3OH) λ_{max} 360 nm (29,336), 639 nm (7,523).

15. Satisfactory microanalyses were obtained for **4**-**7**, elemental percentages of C, H, and N were within 0.4% of theoretical values.
16. Biliverdins **6** and **7** were obtained in quantitative yields. Crystallization from methylene chloride-hexane gave green crystals mp: >300 °C. By ^{1}H NMR (300 MHz, NaOD) analysis OCH_3 groups are missing.
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