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Hepatobiliary Excretion of Dipyrrinone Sulfonates in Mrp2-Deficient (TR^-) Rats

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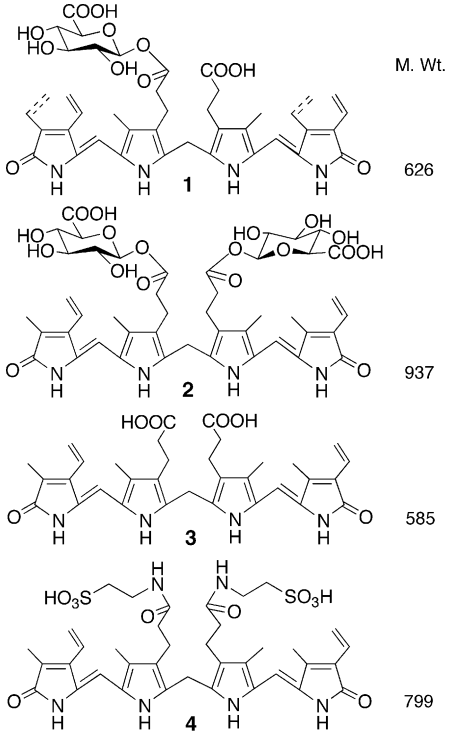
Abstract—The biliary excretion of the sodium salts of 8-(2-ethanesulfonic acid)-3-ethyl-2,7,9-trimethyl-1,10-dihydro-11*H*-dipyrrin-1-one (xanthosulfonic acid) and a fluorescent analogue (8-desethyl-*N*,*N*′-carbonyl-kryptopyrromethenone-8-sulfonic acid) was compared in Mrp2-deficient (TR^-) and normal rats. Both organic anions were excreted rapidly in bile in Mrp2-deficient rats, but the biliary excretion of the fluorescent sulfonate was impaired relative to normal controls. The rat clearly has efficient Mrp2-independent mechanisms for biliary efflux of these anions that are not used by bilirubin or its mono- and diglucuronides.

Efflux from liver to bile is a major process in the elimination of countless endogenous and exogenous chemicals from the body. Taurine and glycine amides of hydroxylated cholanic acids (bile salts), which are the major solutes of bile, are actively transported through the canalicular membrane of hepatocytes by an ATP-powered protein known as BSEP (bile salt export pump).[1] In contrast, the monoglucuronide (**1**) and diglucuronide (**2**) conjugates of bilirubin (**3**) (Scheme 1), which impart to bile its golden hue, are thought to be actively transported across the same membrane by the ATP-ase Mrp2 (multidrug resistance associated protein 2), also known as cMOAT (canalicular multispecific organic anion transporter).[2,3] Mutant rats (TR^- and EHBR rats) that do not express this protein are unable to excrete bilirubin glucuronides efficiently in bile and exhibit mild conjugated hyperbilirubinemia because of the accumulation of bilirubin glucuronides.[4,5] Similarly, people with Dubin–Johnson syndrome, caused by a rare genetic defect in Mrp2 synthesis, also develop hyperbilirubinemia. In addition to bilirubin glucuronides, the biliary excretion of a number of other chemicals has been shown to be impaired in Mrp2-deficient rats.[2,3] These include glucuronide and glutathione conjugates as well as glutathione itself. Such animal studies, along with isolated perfused liver experiments and many



Scheme 1. Linear representations of bilirubin monoglucuronides (**1**), bilirubin diglucuronide (**2**), bilirubin (**3**) and bilirubin ditaurine amide (**4**). (Only one of two possible isomeric monoglucuronides is depicted; in the other isomer, the positions of the methyl and vinyl groups on the end rings are reversed, placing vinyl groups as indicated by dotted lines in **1**.)

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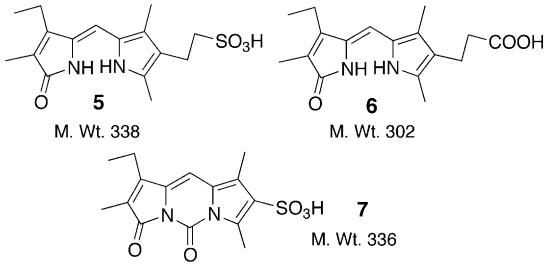
studies using hepatocytes or membrane vesicles in vitro, have led to the current theory that Mrp2 is a general ATP-driven membrane transporter or efflux pump of amphipathic non-bile salt organic anions, essential for their efficient hepatobiliary disposal. There are, however, notable apparent exceptions.[6] One is the disulfonic acid indocyanin green, which seems to depend on multidrug resistance P-glycoprotein 2 (Mdr2), rather than Mrp2, for its efflux into bile.[7] Another is bilirubin (**3**), which is not excreted significantly in bile. And a third is the ditaurine conjugate of bilirubin (**4**).[8] This water-soluble conjugate is not biosynthesized in mammals, but is the principle yellow pigment in the bile of some fish.[9] When administered parenterally to Mrp2-deficient (TR^-) rats or perfused through isolated TR^- rat livers, it is excreted quantitatively and rapidly in bile, just as in normal rats.[8]

The marked differences in hepatobiliary disposal between the three groups of tetrapyrrolic organic anions represented by the ionized forms of bilirubin glucuronides (**1** and **2**), bilirubin (**3**), and ditaurobilirubin (**4**) prompted us to examine the biliary excretion of the sodium salt of xanthosulfonic acid (**5**), a new sulfonated analogue of xanthobilirubinic acid (**6**).[10] Xanthosulfonic acid retains the *Z*-dipyrrinone chromophore of **1**–**4** and the acidic sulfonic acid group of **4**, but lacks the conformational flexibility of tetrapyrroles **1**–**4**. We find that



5, like **4**, is highly cholephilic and is excreted rapidly in bile in TR^- rats. Mrp2 is, therefore, not required for its canalicular excretion. Similar behavior was also observed for **7**, which resembles **5** but belongs to a new class of highly fluorescent dipyrrinones, called xanthoglows.[11] These sulfonated dipyrrinones, particularly xanthoglows, may be useful, uncomplicated compounds for investigating mechanisms of hepatic transport and canalicular efflux of organic anions in vitro.

The biliary excretion of small (~250 μg) intravenous bolus doses of **5** was studied in normal (Sprague–Dawley) rats and in homozygous TR^- and Gunn rats. Homozygous Gunn rats are a mutant, originally Wistar, strain that lacks the ability to glucuronidate bilirubin.[12] Consequently, their bile is devoid of bilirubin glucuronides. We used them for these studies to simplify the appearance and analysis of HPLC chromatograms of bile measured in the 400–450 nm range. In terms of Mrp2 and canalicular transport they are not significantly different from normal rats.

Within minutes of injection, unchanged **5** was detectable in bile.[13] Typical results from an experiment in a Gunn

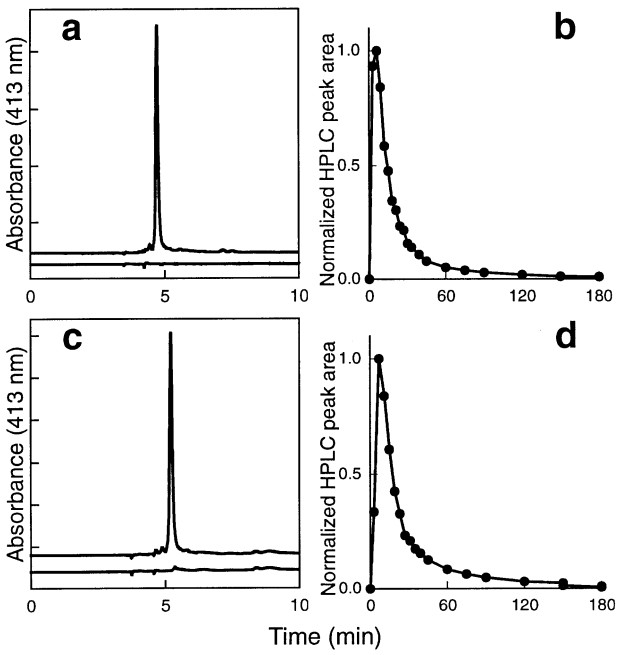


Figure 1. Representative biliary excretion data for **5** in a Gunn (**a**,**b**) and a TR^- rat (**c**,**d**). Panels **a** and **c** show HPLC chromatograms of bile before (*lower line*) and 3 min after (*upper line*) injection of **5**; **b** and **d** are complete excretion curves for each animal. (Note that two different instruments and different batches of eluent were used for HPLC analyses. For this reason, retention times in separate figures, such as in panels **a** and **c**, do not match exactly.)

rat are depicted in Figure 1a and b. Panel **a** shows HPLC chromatograms of bile before (*lower line*) and 3 min after (*upper line*) injection of **5** as a bolus in 1 mL rat serum. The prominent HPLC peak in bile after injection was identified as unchanged **5** by retention time, co-elution with authentic material and its UV/Vis absorption spectrum. No metabolites of **5** absorbing in the 400–450 nm range were detected in bile by HPLC in either Gunn or Sprague–Dawley rats. Panel **b** shows the complete biliary excretion curve for **5** derived from HPLC chromatograms of sequential bile samples. The concentration of **5** in bile increased rapidly, reaching a maximum at ~6 min after injection and then decreased exponentially to very low levels within 60 min. Similar results were seen in Gunn rats and normal rats. In both strains the maximum concentration of **5** in bile greatly exceeded that in the injectate solution, suggesting active secretion. In four animals (2 Gunn and 2 normal) the fraction of the injected dose excreted in bile in 3 h was estimated to be 0.6 ± 0.09. Presumably most of the remainder was excreted in urine. Although we did not monitor urinary excretion of **5**, the compound was detectable in urine samples by HPLC.

Figure 1c and d shows a representative experiment done in a TR^- rat. As in the Gunn and normal rats, unchanged **5** appeared rapidly in bile with a maximum concentration higher than in the injectate. The mean fraction of the injected dose excreted in four animals was estimated as 0.6 ± 0.14, similar to the estimate for Gunn and Sprague–Dawley rats. The biliary excretion profiles for Mrp2-deficient and non-deficient rats were similar (cf. Fig. 1b and d).

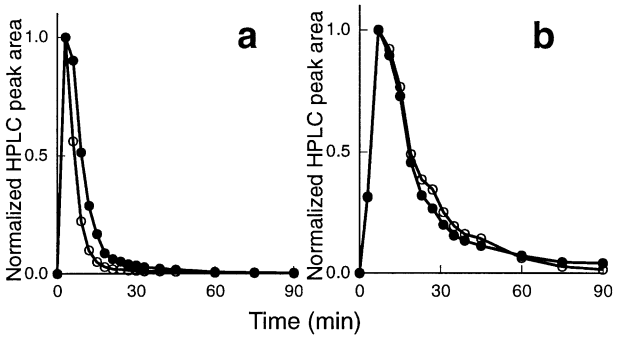
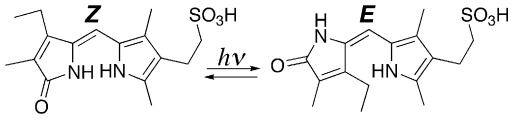


Figure 2. Biliary excretion of **4** and **5** following simultaneous injection into a Sprague–Dawley (**a**) and a TR$^-$ (**b**) rat. Open circles, **5**; closed circles, **4**.

We conclude that sulfonic acid **5** is cholephilic and does not require Mrp2 for efficient excretion into bile. In this respect it behaves like the slightly less polar ditaurine conjugate of bilirubin (**4**). We demonstrated this experimentally by injecting **4** and **5** ($\sim$250 μg of each) simultaneously into a normal Sprague–Dawley rat and a TR$^-$ rat (Fig. 2). The shapes of the biliary excretion curves for each compound were almost identical to each other in both strains. Thus, **5** behaves qualitatively and kinetically like **4** in its biliary excretion, despite the roughly 2-fold difference in molecular weight and charge.

Fluorescent compounds have been useful for investigating hepatobiliary transport mechanisms.[3] Most of those used for studying organic anion transport are complex structures that undergo extensive metabolism and are ultimately secreted in bile as glutathione conjugates. Xanthosulfonic acid (**5**) absorbs strongly at $\sim$413 nm,



Scheme 2. Photochemical isomerization of **5**.

but is not fluorescent, probably because of non-radiative dissipation of excitation energy by $Z \rightarrow E$ configurational isomerization, shown in Scheme 2.[14] $Z \rightarrow E$ isomerization in dipyrrinones can be prevented by covalently joining the two N atoms, which results in a dramatic enhancement of fluorescence.[15] Brower and Lightner have accomplished this using a C=O bridge, producing compounds called xanthoglows which have fluorescence quantum yields close to 1.[11]

The cholephilic behavior of the nonfluorescent **5** led us to study the biliary excretion of the rather similar, slightly more polar, highly fluorescent xanthoglow derivative **7**, which has an emission maximum at 520 nm in MeOH. Figure 3a shows HPLC chromatograms of bile from a Gunn rat before and 3 min after intravenous injection of **7** ($\sim$250 μg) and Figure 3c (*upper curve*) shows the complete biliary excretion profile. Like **5**, **7** was excreted rapidly and without detectable metabolism

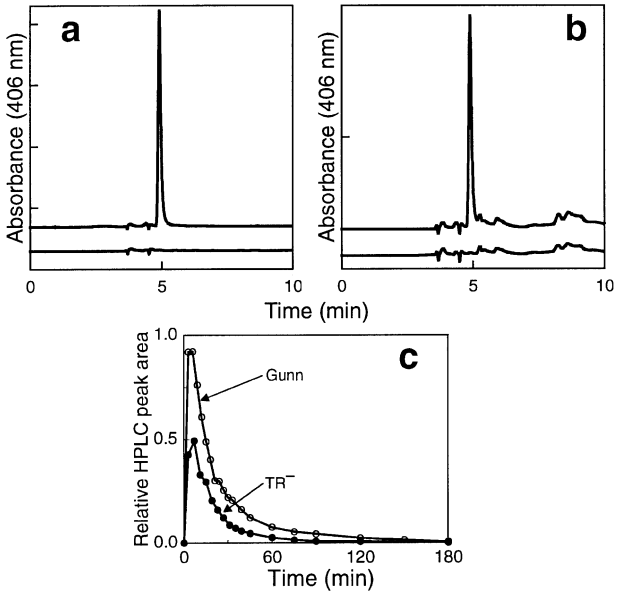


Figure 3. Representative biliary excretion data for **7** in Gunn and TR$^-$ rats. Panels **a** and **b** show HPLC chromatograms of bile before (*lower line*) and 3 min after (*upper line*) injection of **7** into a Gunn and a TR$^-$ rat, respectively. Panel **c** shows complete biliary excretion curves for each animal (derived by plotting HPLC peak areas for **7** in bile normalized to the peak area for **7** in the injectate).

to other pigments with similar UV/Vis absorption. In two animals, the peak concentration in bile was similar to the concentration in the original injectate, suggesting active transport, and the fraction of the dose excreted in bile was estimated as 0.7. Urine and bile collected during the experiments were intensely fluorescent. Xanthoglow **7** was also excreted rapidly in TR$^-$ rats and the biliary excretion profile was similar to that seen in Gunn rats, as shown for a representative experiment in Figure 3b and c. However, the fraction of the injected dose excreted in bile in three TR$^-$ rats was considerably less (0.3 ± 0.09) than in the Gunn rats.

These studies show that both **5** and **7** are taken up rapidly by the liver and that neither of them absolutely requires the presence of Mrp2 (cMoat) for rapid efflux into bile. The absence of the protein had little effect on the biliary excretion of **5**, though it did substantially reduce the biliary excretion of **7**. In view of the similar structures and HPLC mobilities of the two compounds, that difference is surprising. Clearly, there are efficient Mrp2-independent mechanisms in the rat for biliary efflux of the organic anions **4**, **5** and **7** that are not used by bilirubin mono- and diglucuronides or bilirubin itself. The chemistry underlying these Mrp2-independent mechanisms[6,16] is presently unknown, but they could have more general importance for the biliary excretion of anionic drugs and conjugates than the Mrp2-dependent pathway. We hope that by modifying the β-substituents of **7** we will be able to make xanthoglow organic anions that are even better cholephiles and that may be useful for investigating these Mrp-2 independent mechanisms, as well as mechanisms of hepatic uptake and intracellular transport.

Acknowledgements

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13. Full experimental details are given in previous papers.[16–18] Bilirubin ditaurine amide (**4**) was the di-sodium salt from Porphyrin Products (Logan, UT, USA). 8-(2-Ethanesulfonic acid)-3-ethyl-2,7,9-trimethyl-1,10-dihydro-11*H*-dipyrrin-1-one (xanthosulfonic acid, **5**) and 8-desethyl-*N*,*N*′-carbonyl-kryptopyrromethenone-8-sulfonic acid (**7**) were the sodium salts, synthesized and characterized as described elsewhere,[10,11] with λ_{max} in the methanolic HPLC eluent of 413 and 406 nm, respectively. Sprague–Dawley rats were from local suppliers and Gunn and TR^- rats from our own colonies. For excretion studies, the femoral vein and common bile duct of an adult male (>250 g) were cannulated under ketamine anesthesia and a liposomal solution containing phosphatidylcholine (1.5 g), cholesterol (62 mg), sodium cholate (12.95 g) and taurine (3.75 g) in 1 L of water was infused (2 mL/h) through the femoral catheter to maintain hydration and bile flow. The total length of the biliary cannula was 7.5 cm. The animal was placed in a restraining cage under an infrared heating lamp and, once bile flow and body temperature were stable (~30–60 min), pigment (0.25 mg), dissolved in normal rat serum (1 mL) with the aid of a small volume (0.1 mL) of DMSO, was infused via the femoral vein as a bolus over a period of 20–45 s. Bile was collected in 20-μL aliquots into micropipettes from the tip of the bile duct cannula immediately before injection of pigment, 3 min later, and at frequent intervals thereafter. Samples were flash frozen immediately in dry-ice, then kept at −70 °C until analyzed by HPLC. Collection of each bile sample took <1 min for Sprague–Dawley and Gunn rats, but often exceeded 1 min for TR^- rats because of their relatively slow bile flow rates. Consequently, the biliary excretion curves for experiments in TR^- rats are less precise than those for the other two strains. Bile flow rates were measured gravimetrically by periodically collecting timed 3-min aliquots of bile into tared Pasteur tubes. For HPLC, frozen bile samples (20 μL) were mixed with 80 μL of ice-cold 0.1 M methanolic di-*n*-octylamine acetate, microfuged for 30 s, and the supernate (20 μL) was injected onto the column. Isocratic HPLC analyses were run using a Beckman-Altex ultrasphere-IP 5 μm C-18 ODS column (25 × 0.46 cm) fitted with a similarly-packed precolumn (4.5 × 0.46 cm) and Hewlett-Packard multi-wavelength diode array detector. Compounds **5** and **7** were monitored at their absorbance maxima and peak areas measured using HP ChemStation software. The elution solvent was 0.1 M di-*n*-octylamine acetate in 8% aqueous methanol, flow rate 0.75 mL/min, and column temperature ~34 °C. Biliary excretion curves were derived by plotting integrated HPLC peak areas, normalized to the maximum peak area (Figs 1 and 2) or normalized to the peak area of the injectate (Fig. 3), against time. The fraction of the injected dose excreted was estimated by comparing the area under the biliary excretion curve (HPLC peak area versus time), adjusted for total bile volume excreted, with the HPLC peak area of the pigment in a 20-μL sample of the original serum solution injected into the rat. Areas under biliary excretion curves were determined by the trapezoidal method using Un-Scan-It software (Silk Scientific, Inc., Orem, UT, USA). Except for the animal surgery, all procedures were done under orange or red safelights in a darkroom.
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