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An Enzymatic Synthesis of Glucuronides of Azetidinone-based Cholesterol Absorption Inhibitors

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Abstract—Two derivatives, **1** and **3**, of a novel cholesterol absorption inhibitor, Sch 58235, were glucuronidated with the help of glucuronyl transferases derived from bovine and dog liver microsomes. An efficient procedure for the iodination of **4** was developed on an analytical scale to be used for the preparation of a ^{125}I-labeled radioactive glucuronide **5**.

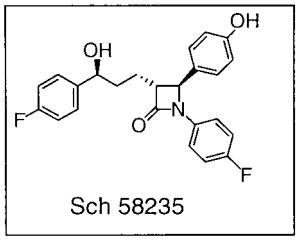
Introduction

Glucuronidation, the most common pathway for elimination of xenobiotics, is catalyzed by a family of glucuronyl transferases (GT) that transfer the glucuronyl moiety from uridine 5′-diphosphoglucuronic acid (UDPGA) to the appropriate acceptor.[1] The attachment of this highly hydrophilic moiety increases the solubility of a parent molecule facilitating its detoxification by excretion. Moreover, glucuronidation of drug substances may affect their pharmacological activity[2], as well as pharmacokinetics and/or the pharmacodynamics. It is not surprising, therefore, that in vitro synthesis of glucuronidated derivatives of drug candidates often becomes of primary importance to metabolic studies.

The success or failure of a chemical synthesis of a glucuronide primarily depends on the structural complexity and the chemical stability of the molecule of interest. However, even in the case of relatively simple and stable compounds, chemical glucuronidation is an involved and relatively low yielding process that requires several protection and deprotection steps.[3,4]

Considering that a variety of naturally occurring glucuronyl transferases having a broad substrate specificity are readily available from a variety of sources,[5] and that the biological glucuronidation occurs under mild conditions without requiring any protective steps, the enzymatic synthesis of glucuronides becomes an attractive alternative (for in vitro enzymatic glucuronidations see refs. 6–16).

During the course of metabolic studies of a novel cholesterol absorption inhibitor, Sch 58235, there was a demand for milligram quantities of fluorescent glucuronide analogue **2** and ^{125}I labeled glucuronide **5**.[17] It was decided to synthesize **2** by direct glucuronidation of **1** in the presence of UDPGA and a transferase. In the case of a photoaffinity derivative **5**, it was suggested to synthesize the glucuronide of the tributylstannyl derivative **4** first and then iodinate it with Na^{125}I in order to minimize the handling of radioactively labeled material. Below we describe the enzymatic synthesis of glucuronides **2** and **4** as well as the application of a simple iodination procedure that can be utilized for the synthesis of radioactively labeled ^{125}I-(**5**).



Sch 58235

Results and Discussion

The enzymatic transformation of **1** was investigated first. Since the structure of Sch 58235 and Sch 60672 are

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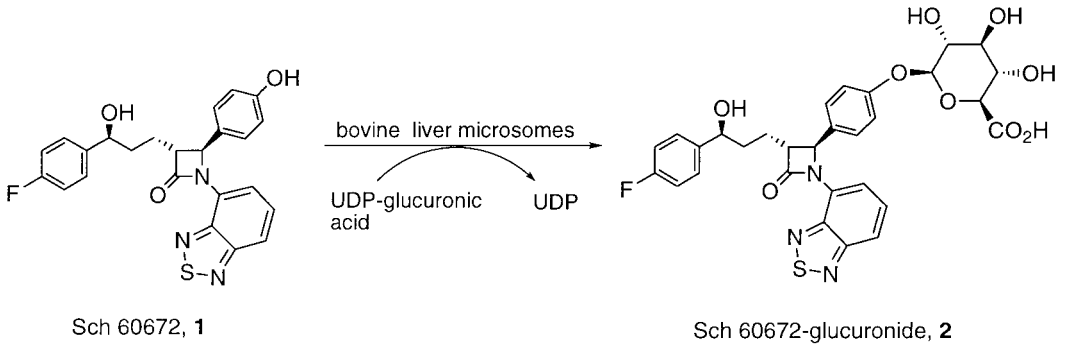
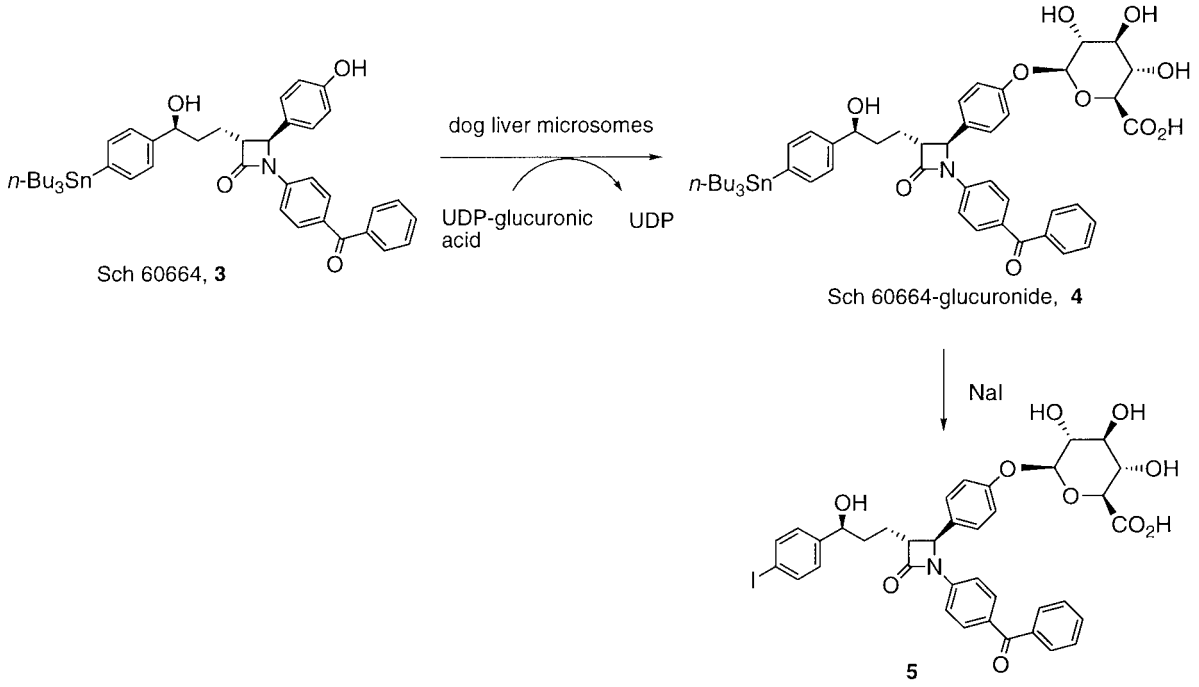


Scheme 1.



Scheme 2.

similar, the reaction conditions developed for the synthesis of Sch 58235-glucuronide[18] were adopted for the synthesis of **2**. The glucuronidation was carried out under nitrogen atmosphere in Tris buffer in the presence of an excess of UDPGA (it was found that a large excess of UDPGA is required to complete the transformation because of enzymatic hydrolysis of UDPGA by impurities in the crude commercial preparation of the transferase). After a 5 h incubation 95% of **1** was converted to the corresponding glucuronide **2**. Following the purification, 7.7 mg of **2** (53% yield) was isolated.

Encouraged by the initial results, we applied the above procedure to glucuronidation of **3**. To our disappointment no reaction took place. Most likely, the failure of **3** to react was due to the presence of bulky tributylstannyl and benzophenone moieties that prevented the proper orientation of the phenyl hydroxyl into the active site of the transferase. The attempt to utilize commercially available rabbit liver microsomes was similarly unsuccessful. The addition of 3–5% ethanol to the reaction medium to increase the solubility of Sch 60672 has not improved the yield either.

Since commercially available GT preparations failed to catalyze the glucuronidation of **3**, we turned to other sources: four additional non-commercial GT preparations including microsomes obtained from monkey, dog, and rat livers and rabbit intestinal mucosa were tested. It was also decided to introduce 0.1% of surfactant into the reaction medium, since some surfactants are known to activate GT.[19] The transformations were carried out under standard conditions (see Materials and Methods) for 18 h, after which samples were withdrawn, diluted 5-fold with ethanol to stop the reaction and analyzed for product formation by HPLC. The results presented in Table 1 indicate that 0.1% of either digitonin or Triton X-100 stimulated the activity of bovine and rabbit GT, albeit to a small extent. No product formation was observed in the samples containing microsomes from rabbit intestinal mucosa, or from rat or monkey liver. Most rewarding, however, was the fact

Table 1. Enzymatic glucuronidation of **3**[a]

Microsomal preparation (mg/mL)	Surfactant	Conversion 2 h, %	Conversion 18 h, %
Bovine liver (75)	None	0	0
	D[b]	< 0.5	3.1
	T[c]	0.5	0.5
Rabbit liver (75)	None	0	0
	D	< 0.5	0.73
	T	< 0.5	0.5
Dog liver (2.9)	None	4.0	29.7
	D	0.9	4.1
	T	2.8	18.1

[a] For conditions see Materials and Methods.
[b] D-digitonin, 0.1%.
[c] T-Triton X-100, 0.1%.

that dog liver microsomes exhibited an appreciable activity resulting in 30% conversion of **3** within 18 h. It was also apparent that neither surfactant was required for activity of dog liver GT, a positive feature since surfactants often complicate product isolation and purification.

In an attempt to optimize the reaction conditions, the effect of mercaptoethanol (which is known to activate the bovine GT)[20] was examined. To our surprise, mercaptoethanol deactivated dog liver GT. The presence of 5 mM of mercaptoethanol in the reaction medium resulted in up to 5-fold reduction of the glucuronide yield.

The reaction conditions outlined in Materials and Methods were used for the glucuronidation of the remaining 2.6 mg of **3** (due to the complexity of the synthesis, very limited quantities of **3** were available). After 48 h, 40% of **3** was converted into the corresponding glucuronide **4** which was isolated (0.93 mg, 30% yield).

In order to develop an iodination procedure to be later used with the radioactive iodine, the standard iodination protocol[21] was adopted for the iodination of **4** on a 50 nmol scale. The iodination of **4** with $Na^{127}I$ on a solid phase matrix of Iodogen was complete after a 10 min incubation at 23°C as indicated by complete disappearance of the stannyl derivative **4** as shown by HPLC. The product, purified by reversed phase HPLC, was positively identified by MS as **5**.

In conclusion, glucuronides **2**, **4** and **5** were synthesized with the help of glucuronyl transferases derived from two unrelated sources. An efficient procedure for the iodination of **4** was developed on an analytical scale to be used for the preparation of a photoaffinity labeled radioactive glucuronide ^{125}I-(5).

Materials and Methods

Uridine 5′-diphosphglucuronyl transferase from bovine liver (Type III) and rabbit liver (Type II) were purchased from Sigma Chemical Company (St. Louis, MO) as lyophilized powders with a specific transferase activity of 2.2 and 2.8 units/g respectively. Microsomes from monkey, dog, and rat livers and from rabbit intestinal mucosa were kindly provided by Dr. Nathan Yumibe (SPRI). Mercaptoethanol, dithiothreitol (DTT), Triton X-100, UDP-glucuronic acid ammonium salt were also obtained from Sigma. IODO-GEN® reagent was bought from Pierce, Rockfort, IL. All other salts and solvents were purchased from Fisher (Fair Lawn, NJ) and were of the highest purity available.

1-*O*-[4-[1-(2,1,3-benzothiadiazol-4-yl)-3(*R*)-[3(*S*)-hydroxy-3-(4-fluorophenyl)propyl]-2-oxo-4(S)-azetidinyl]phenyl]-β-D-glucuronic acid (2). Bovine live transferase (800 mg) was added to 100 mM Tris buffer (10 mL, pH 8.4) containing $MgCl_2$ (40 mM) and DTT (10 mM). A solution of **1** (10 mg) in THF (0.3 mL) was added, and the formed suspension was stirred for 5 min prior to the addition of UDPGA ammonium salt (100 mg). The mixture was shaken under N_2 for 5 h. The microsomes were precipitated by the addition of ethanol (10 mL) and removed by centrifugation. The pH was adjusted to 2 with 6 M HCl and the glucuronide was extracted first into EtOAc and then into aqueous bicarbonate. The pH of the bicarbonate solution was lowered to 2 and the glucuronide was extracted back into EtOAc. It was then purified by preparative TLC (EtOAc/MeOH/AcOH: 60/40/2), extracted from silica with MeOH and the solvent evaporated. The solid was dissolved in an aqueous bicarbonate (pH ~9), then neutralized to pH 2 and extracted with EtOAc. The organic phase was dried with Na_2SO_4, and evaporated under vacuum to give 7.7 mg of the glucuronide. MS analysis: calcd for $(M+Na)^+$ $C_{30}H_{28}FN_3O_9SNa$ M 648.1428; found 648.1430.

1-*O*-[4-[1-(4-benzoylphenyl)-3(*R*)-[3(*S*)-hydroxy-3-[4-tributylstannyl)phenyl]propyl]-2-oxo-4(*S*)-azetidinyl]phenyl]-β-D-glucuronic acid (4). To 150 mM of Tris buffer pH 7.9 (3 mL) containing $MnCl_2$ (2 mM), and dog liver microsomes (15 mg), ethanol solution (0.22 mL) of **3** (2.6 mg) was added. The reaction was initiated by the addition of UDPGA ammonium salt (25 mg) and allowed to proceed at 34°C at continuing shaking for 40 h at which point the degree of conversion reached 40%. The reaction mixture was concentrated to ~1 mL on a Speed-Vac concentrator. Ethanol (4 mL) was then added and the mixture was sonicated for 5 min. The resulting suspension was clarified by centrifugation and further concentrated to ~0.5 mL. The sample was then purified by reversed phase HPLC on a YMC ODS–AM column (3 μm, 4.6×100 mm) using a gradient of acetonitrile and aqueous ammonium acetate buffer. MS analysis: calcd for $C_{49}H_{61}NNaO_{10}Sn$. $(M+Na)^+$ 964.33; found 964.

1-*O*-[4-[1-(4-benzoylphenyl)-3(*R*)-[3(*S*)-hydroxy-3-(iodophenyl)propyl]-2-oxo-4(*S*)-azetidinyl]phenyl]-β-D-glucuronic acid (5). An aliquot (0.15 mL, 530 nmol) of Iodogen solution in CH_2Cl_2 placed into 0.5 mL Eppendorf tube and the solvent was evaporated under a stream of N_2. To this tube 0.1 mL of 20 mM ammonium bicarbonate buffer (pH 7.8) and 0.05 mL of the solution

of **4** in ethanol (53 nmol) were added. Following the addition of 0.008 mL of 1 mg/mL solution of NaI in water (53 nmol) the reaction was vortexed for 10 min at 23°C. The iodinated product **5** was purified by reversed phase HPLC on a YMC ODS–AM column (3 μm, 4.6×100 mm)) using a gradient of acetonitrile and aqueous ammonium acetate buffer. MS analysis: calcd for $C_{37}H_{35}INO_{10}$ $(M+H)^+$ 780.57; found 780.

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