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Enzymatic Glucuronidation of a Novel Cholesterol Absorption Inhibitor, SCH 58235

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ABSTRACT

A glucuronide of a novel cholesterol absorption inhibitor was synthesized on a 200-mg scale in one step via bovine liver glucuronyltransferase-catalyzed coupling of the glucuronyl moiety of UDP-glucuronic acid with the phenolic hydroxyl of Sch 58235. It was shown that the product yield is limited by the hydrolysis of UDP-glucuronic acid by impurities present in the commercial microsomal preparation of the transferase. This detrimental effect of UDPGluA hydrolysis could be diminished by the presence of high concentration of glucuronlytransferase. Optimization of reaction conditions and purification procedure resulted in a process that proceeded with 95% conversion and 88% isolated product yield. The $^{13}C_6$-glucuronide of Sch 58235 was prepared with the help of a cascade of eight enzymes operating concurrently in one pot.

Index Entries: Glucuronidation; uDP-glucuronyltransferase; Sch 58235; cholesterol absorption inhibitor.

Abbreviations: UDPGluA, uridine 5′-diphosphoglucuronic acid; GT, glucuronyltransferase; DTT, dithiothreitol.

INTRODUCTION

Glucuronidation is the most common pathway for detoxification and elimination of hydrophobic xenobiotics occurring in tissues of most mammals *(1)*. The reaction is catalyzed in vivo by a family of glucuronyltransferases that transfer the glucuronyl moiety from UDPGluA, a ubiquitous compound in cells, to the appropriate acceptor (phenol, alcohol, carboxyl, amine, amide, and so on), with inversion of stereochemistry at the

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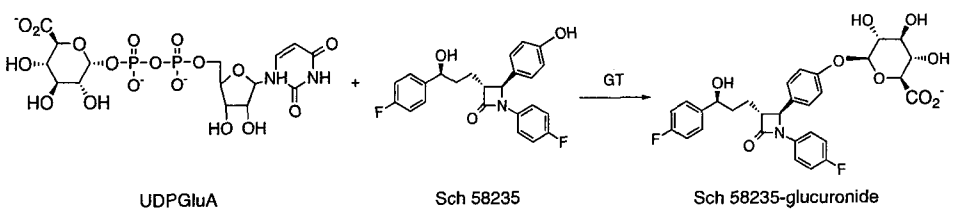
anomeric carbon. This attachment of a highly hydrophilic moiety significantly increases solubility of the conjugates, and thereby facilitates their detoxification by excretion. Enhanced detoxification, however, is not the only outcome of the glucuronidation process. It has been shown, for example, that glucuronidation of morphine at the 6-position converts it into a much more potent opiate-receptor agonist than morphine itself. However, glucuronidation of morphine at the 3-position makes it an extremely potent antagonist *(2)*. Because of their ubiquitous nature and high physiological significance, development of an efficient in vitro synthesis of glucuronides often becomes critical during studies of drug metabolism undertaken in the development of a new pharmaceutical product.

Commonly used chemical synthesis of glucuronides *(3,4)* is a multistep, relatively low-yielding process that involves several protection and deprotection steps. In the case of labile compounds, the use of chemical methods often fails to produce even a minimal amount of the desired glucuronides.

An alternative synthesis of glucuronides is based on in vitro utilization of glucuronyltransferases (GT) in isolated form, or as a part of intact microsomes. GTs are membrane-bound enzymes present in many tissues *(5)*. Their activity strongly depends on the presence of phospholipids, and disappears almost completely when the phospholipids are removed. Because GTs participate in elimination of a large number of xenobiotics, most of them have a broad substrate specificity.

Numerous reports describe the application of GT to the synthesis of glucuronides. For example, microsomes from a variety of sources have been used for glucuronidation of naproxen, pirprofen, flurbiprofen, ibuprofen, banoxaprofen, labetalol, and other phenolic compounds *(6–11)*. GT immobilized on alginate beads was utilized for the synthesis of glucuronides of AZT, bilirubin, and morphine *(12)*. GT from rabbit liver found its application in the synthesis of fluorescein monoglucuronide *(13)*. Enzymatic synthesis of glucuronides of several lipophilic compounds was also carried out in a hollow-fiber membrane reactor *(14)*.

Considering the relative complexity of chemical synthesis *(15)* and the wide availability of glucuronyltransferases from various sources, the authors set out to develop an efficient enzymatic method for the synthesis of $^{12}C_6$- and $^{13}C_6$-glucuronides of Sch 58235, a novel cholesterol absorption inhibitor (Scheme 1).



Scheme 1. Enzymatic synthesis of Sch 58235-glucuronide.

MATERIALS AND METHODS

Uridine 5'-diphosphoglucuronyltransferase (Type III) was purchased from Sigma (St. Louis, MO) as a lyophilized powder with a specific activity of 2.2 U/g. ATP, UTP, NAD, 1,6-glucose-diphosphate, pyruvate, phosphoenolpyruvate, mercaptoethanol, UDP-glucuronic acid ammonium salt, glucokinase (from *Bacillus stearothermophilus*), phosphoglucomutase (from rabbit muscle), UDP-glucose pyrophosphorylase (from bovine liver), UDP-glucose dehydrogenase (Type III, from bovine liver), lactate dehydrogenase (Type II, from rabbit muscle), inorganic pyrophosphatase (from bakers yeast), and pyruvate kinase (Type II, from rabbit muscle) were also purchased from Sigma. All other salts and solvents were purchased from Fisher (Fair Lawn, NJ), and were of the highest purity available. D-Glucose 99%+ chemical purity (U-$^{13}C_6$, 98–99%) was obtained from Cambridge Isotope Laboratories (Andover, MA). The course of glucuronidation reactions was followed by HPLC equipped with Zorbax ODS (0.46 × 25 cm) column. Buffer A, 50 m*M* ammonium acetate, pH 6.0; buffer B, 100 acetonitrile. Gradient: 70% buffer A for 5 min, then linear to 90% buffer B in 25 min; hold for 10 min. Flow rate, 1 mL/min. Retention times: glucuronide, 7.9 min; Sch 58235, 23.4 min. Detection, UV 245 nm.

Synthesis of Sch 58235-Glucuronide Under Optimized Conditions

To 210 mL of 250 m*M* Tris buffer, pH 8.0, containing 10 m*M* mercaptoethanol, 40 m*M* $MgCl_2$, and 17.5 g glucuronyltransferase from bovine liver. 220 mg of Sch 58235 in 6.6 mL of THF were added, and the mixture was incubated for 10 min under N_2 at 23°C. The reaction was initiated by the addition of 2.0 g of UDPGluA dissolved in 10 mL of the Tris buffer. The reaction was maintained under N_2 at 23°C, reaching 95% conversion after 6.5 h or incubation.

Isolation

The glucuronide was isolated by adjusting the pH of the reaction medium to 2.0 with HCl, followed by extraction with ethyl acetate. The glucuronide was then recovered by back-extraction with aqueous sodium carbonate. This was acidified to pH 2.0, and extracted with ethyl acetate again. This procedure was repeated 3×. The glucuronide was then purified further by preparative TLC R_f = 0.35 (EtOAc:MeOH:AcOH [80:20:2.0]).

Analysis

Sch 58235-glucuronide high resolution MS(FAB): Calculated for $(M+H)^+$ $C_{30}H_{30}NO_9F_2$, 586,1889, found 586,1874; $^{13}C_6$-Sch 58235-glucuronide: LC-ESI/MS/MS calculated for (M-H)-590.19, found 590.2.

RESULTS

GT-Catalyzed Glucuronidation of Sch 58235

The ability of bovine liver GT to glucuronidate Sch 58235 was tested under a modified Fishman and Bernfield procedure *(16)* as described in Sigma technical protocols. Five mg of Sch 58235 were dissolved in 0.27 mL of ethanol, and the solution added to 9.7 mL of 250 m*M* Tris buffer containing 42 m*M* $MgCl_2$, 2.8 m*M* DTT, 2.1 m*M* UDPGluA, and 200 mg of the transferase. The mixture was incubated at 23°C. The product accumulated for the first 2.5 h, and then stopped at approx 3.0% conversion. Since Sch 58235 has a very low solubility in water, it was decided to investigate the effect of an organic co-solvent on the efficiency of the glucuronidation reaction. Among the solvents tested for their ability to dissolve or emulsify Sch 58235 in Tris buffer, THF was found to be the most efficient. Therefore, the effect of different concentrations of THF on the reaction was investigated. The sharp dependence of conversion on the THF concentration presented in Fig. 1 illustrates the importance of the choice of co-solvent: In the presence of 3% THF, glucuronide yield is sixfold higher than that without a co-solvent. However, higher concentrations of THF have a detrimental effect on yield, presumably caused by inactivation of the catalyst.

Regardless of the concentration of the co-solvent, the reactions stopped after about 4 h, even though more than 50% of Sch 58235 remained unconverted. It was hypothesized that enzyme inactivation and/or hydrolysis of UDPGluA might have contributed to the low yield. In order to identify the actual factor responsible for low conversion, another reaction was set up under the above conditions, and run for 4 h. When it reached plateau at 25% conversion, the content of the reaction was divided equally into three vials. UDPGluA (4.2 m*M*) was added to vial 1, 30 mg/mL of the transferase to vial 2, and both UDPGluA and the transferase to vial 3. After 4 h of incubation at 23°C, glucuronide yield in all three vials was measured. Although it was found to remain unchanged in vial 2, the yield increased by 10 and 15% in vials 1 and 3, respectively. This result made it apparent that the enzyme inactivation was not responsible for low product yield, but rather the disappearance of UDPGluA was the problem. This conclusion was later confirmed directly by measuring UDPGluA concentration during the course of incubation with the transferase. Under the standard reaction conditions (in the absence of Sch 58235), UDPGluA concentration decreased by a factor of 50 in less than 2 h.

In an attempt to alleviate this undesirable side reaction, the rate of hydrolysis of UDPGluA was measured as a function of pH (Fig. 2), with hope that the pH optima for glucuronidation and hydrolysis reactions would be different. Unfortunately, the maximum rate for both the reac-

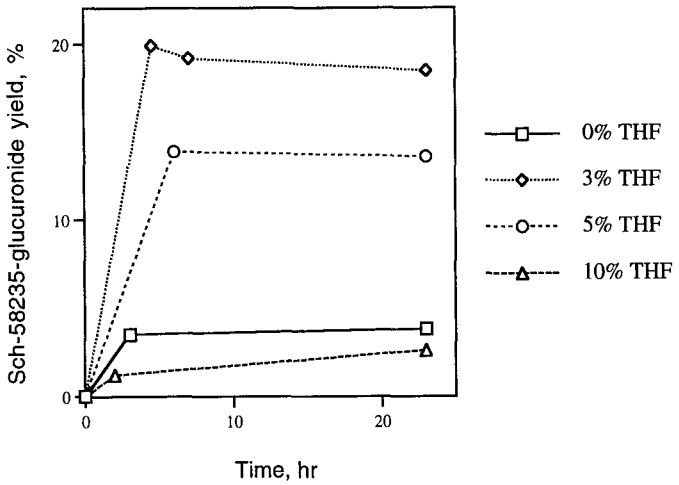


Fig. 1. Effect of THF concentration on yield of Sch 58235-glucuronide (*see* Results and Discussions for conditions).

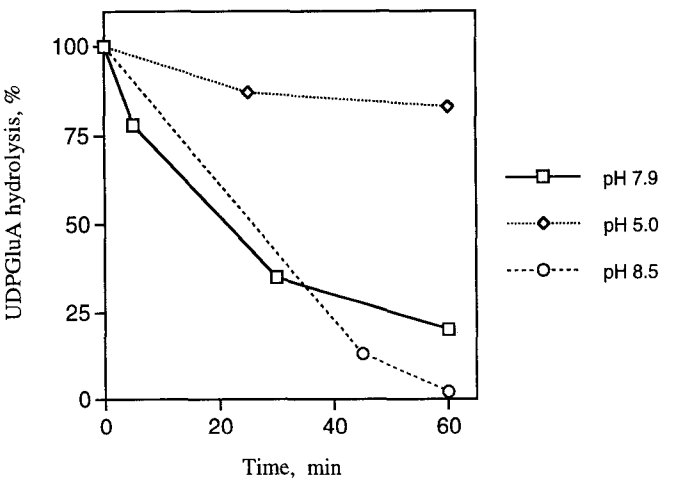


Fig. 2. pH dependence of UDPGluA hydrolysis (conditions: 250 m*M* Tris-HCl buffer, 42 m*M* $MgCl_2$, 2.8 m*M* DTT, 2.0 m*M* UDPGluA, 20.0 mg/mL transferase from bovine liver, 25°C.

tions were found to be above pH 8.0, and therefore the elimination of UDPGA hydrolysis by carrying the glucuronidation at low pH was not possible.

Improvement in glucuronide yield, however, was achieved by increasing the transferase concentration in the reaction medium (Fig. 3). As the concentration of transferase was increased from 10 to 35 mg/mL, the product yield doubled. This unusual result could be explained by assum-

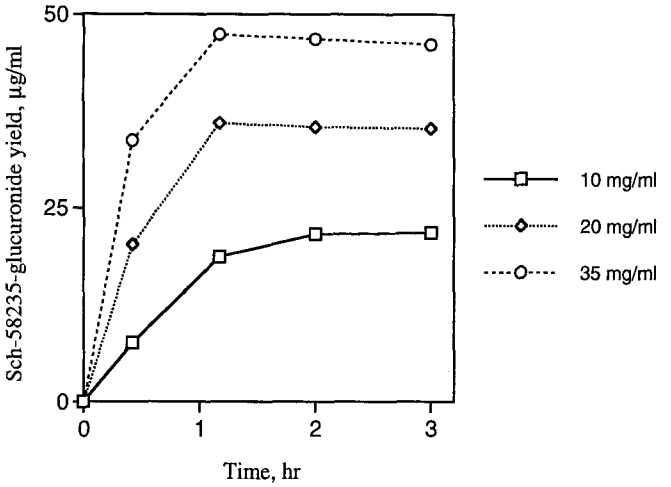


Fig. 3. Glucuronide yield as a function of transferase concentration (conditions: 42 m*M* $MgCl_2$, 2.8 m*M* DTT, 2.0 m*M* UDPGluA, 0.5 mg/mL Sch 58235, 23°C).

ing the presence of an inhibitor of the UDPGluA-hydrolyzing enzyme in the crude preparation of the transferase. In the case of an enzyme preparation that contains a reversible inhibitor, the percentage of the enzyme in the inactive form increases as the concentration of the inhibitor in the incubation mixture increases. Therefore, at a high transferase concentration, the rate of UDPGluA hydrolysis would be partly inhibited. As a consequence, relative rate of the side reaction, compared to the glucuronidation reaction, would diminish, leading to increased yield. It should be mentioned, however, that although this explanation is plausible, other possibilities, including concentration-dependent glucuronyltransferase activation, cannot be excluded.

By taking into account the aforementioned findings, conditions for the glucuronidation were optimized (*see* Materials and Methods), and the reaction was carried out on a 220-mg level. After about 6 h of incubation at 23°C, 95% conversion was achieved (Fig. 4). The product was removed from the reaction medium by acidification to pH 2.0, and extraction with ethyl acetate. The glucuronide was purified by repeated partitioning between ethyl acetate and aqueous sodium carbonate, as described in Materials and Methods, to give 290 mg of pure material (88% yield).

Synthesis of Sch 58235-$^{13}C_6$-Glucuronide

Since $^{13}C_6$-UDPGluA is not available commercially, $^{13}C_6$-glucose was used as a starting material for the synthesis of $^{13}C_6$-labeled derivative of Sch 58235-glucuronide (Scheme 2; similar multienzyme systems used for sialylation, fucosylation, and synthesis of LacNAc have been previously

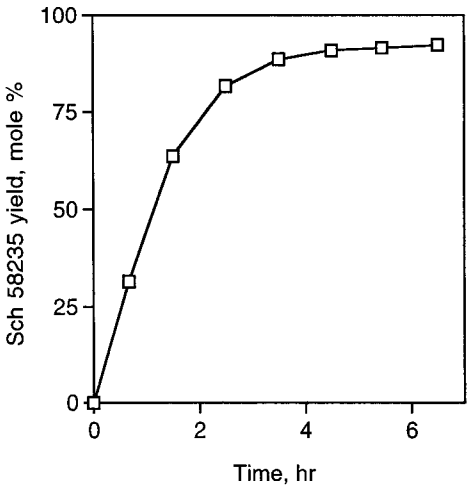
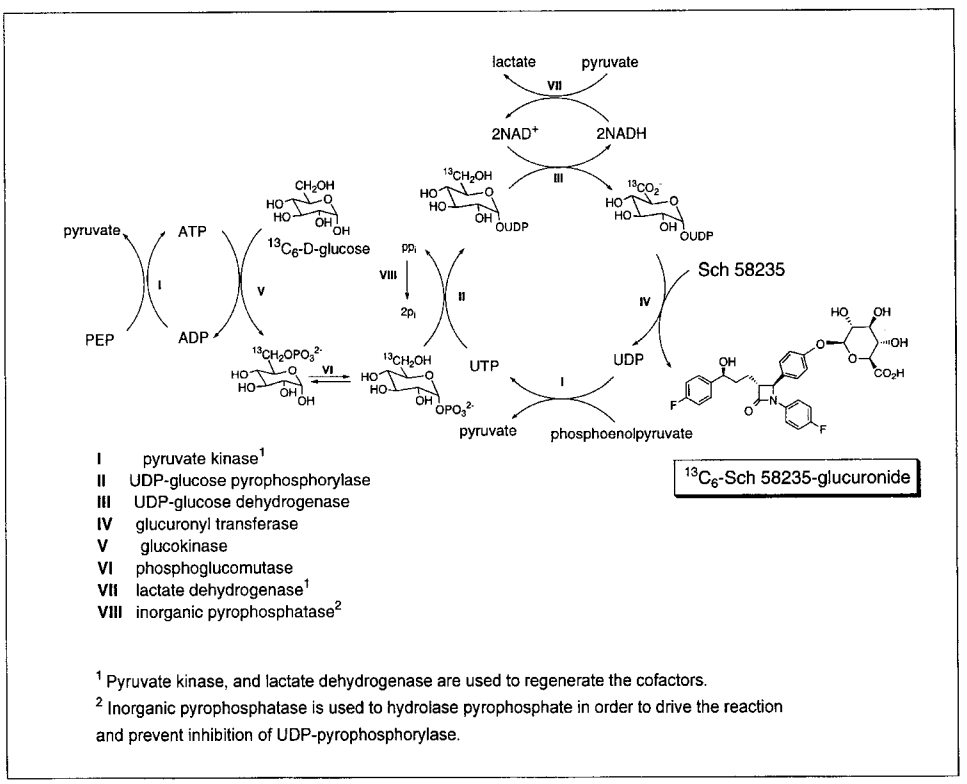


Fig. 4. Synthesis of Sch 58235-glucuronide under optimized conditions (*see* Materials and Methods for detail).



Scheme 2. Enzymatic synthesis of $^{13}C_6$-Sch 58235-glucuronide.

Table 1
Components of Multienzymatic System for the Synthesis of $^{13}C_6$-Sch 58235-glucuronide

#	Component	Activity/ concentration
1	Tris buffer, pH 8.0, 100 m*M*	6.0 mL
2	Glucose	11.1 m*M*
3	ATP	5.0 m*M*
4	UTP	24.0 m*M*
5	NAD	2.8 m*M*
6	Pyruvate	11.0 m*M*
7	Glucokinase	8.0 U
8	Phosphoglucomutase	48.0 U
9	1,6-glucose-diphosphate	0.02 m*M*
10	UDP-glucose pyrophosphorylase	15.0 U
11	UDP-glucose dehydrogenase	5.0 U
12	Lactate dehydrogenase	150.0 U
13	PEP	11.0 m*M*
14	$MgCl_2$	20 m*M*
15	Inorganic pyrophosphatase	75 U
16	Pytuvate kinase	250 U
17	Mercaptoethanol	7.0 m*M*
18	UDPGA-transferase	0.78 U
19	THF	3%
20	SCH 58235	5.2 m*M*

described: see ref *17–20*). As illustrated in this scheme, the $^{13}C_6$-glucose is first converted to glucose-6-phosphate with glucokinase, and then isomerized to glucose-1-phosphate with phosphoglucomutase. Following the condensation with UDP catalyzed by UDP-glucose pyrophosphorylase, the resulting UDP-glucose is oxidized to UDP-glucuronic acid by UDP-glucose dehydrogenase and NAD^+. Finally, the $^{13}C_6$-glucuronyl moiety is transferred to the phenyl hydroxyl of Sch 58235 with the help of glucuronyltransferase. The co-factor regeneration is accomplished with pyruvate kinase and lactate dehydrogenase. To drive the reaction forward and to prevent the inhibition of UDP-glucose pyrophosphorylase, the pyrophosphate formed during the cycle is hydrolyzed by inorganic pyrophosphatase.

An independent experiment determined that it was advantageous first to accumulate $^{13}C_6$-UDPGluA and then to let it react with Sch 58235. Therefore, the transformation was carried out in two steps. To 6.0 mL of 100 m*M* Tris buffer, components 1–16 (Table 1) were first added and incu-

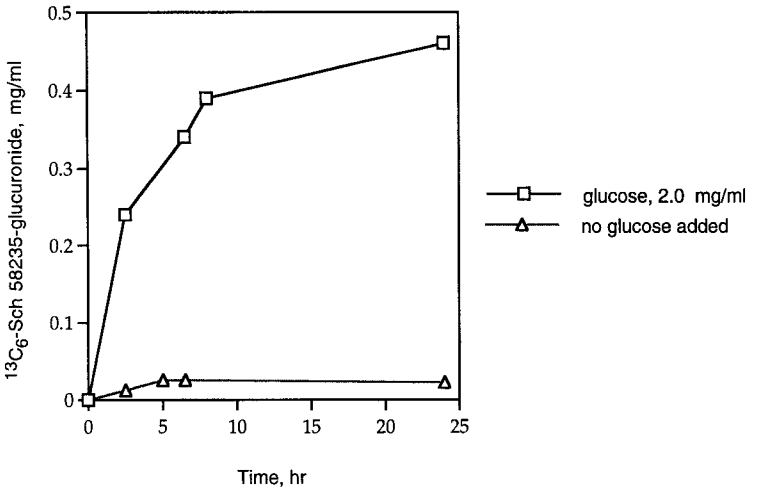


Fig. 5. Synthesis of Sch 58235-$^{13}C_6$-glucuronide (*see* Results and Discussion and Table 1 for detail).

bated at 23°C, with stirring under nitrogen. The pH was maintained at 8.3 by the automatic addition of 1.0 *M* NaOH with a pH-stat. After 20 h, components 17–20 were added, and the reaction was allowed to proceed for another 25 h at pH 8.0. The course of the reaction is presented in Fig. 5. The reaction mixture was acidified to pH 2.5 with 6 *M* HCl, extracted with EtOAc, and purified by preparative TLC (EtOAc:MeOH:AcOH [70:30:2]), to give 2.5 mg of pure $^{13}C_6$-Sch 58235-glucuronide.

CONCLUSIONS

Recent metabolic studies carried out on animal models indicate that, immediately following intake, Sch 58235 is converted to a glucuronyl derivative. In order to determine the drug tissue distribution, isotopically labeled glucuronide derivatives of Sch 58235 had to be synthesized. The chemical synthesis of unlabeled Sch 58235-glucuronide was accomplished using glucuronic acid *(21)*, but this procedure could not be applied to the synthesis of the ^{13}C-labeled analog, because ^{13}C-glucuronic acid is not commercially available. Since it is known that a variety of hydrophobic xenobiotics are efficiently glucuronidated in vivo, a direct biocatalytic route, based on glucuronyltransferases, was undertaken. The synthesis of the unlabeled glucuronide was accomplished in one step, with the help of GT from bovine liver. It has been found that product yield strongly depends on the concentration of the co-solvent, UDPGluA, and the transferase. It has been determined that UDPGluA is hydrolyzed by impurities in the GT preparation, and that the rate of hydrolysis relative to glucuronidation can be diminished by increasing concentration of GT in the reaction medium.

Following optimization of reaction conditions, degree of conversion was increased from 3 to 95%, $^{13}C_6$-derivative of Sch 58235-glucuronide was then prepared with the help of a cascade of eight enzymes operating concurrently in one pot.

ACKNOWLEDGMENTS

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