*Tetrahedron* Vol. 47, No. 28, pp. 5119-5122, 1991
Printed in Great Britain

0040-4020/91 $3.00+.00
Pergamon Press plc

# ENZYMATIC SYNTHESIS OF β-D-GLUCURONIDES WITH *IN SITU* REGENERATION OF URIDINE 5'-DIPHOSPHOGLUCURONIC ACID

**Daniel Gygax*[1], Peter Spies[1], Tammo Winkler[2] and Ulrike Pfaar[1]**

Research and Development Department, Pharmaceuticals Division[1] and Physics Department[2] Ciba-Geigy Limited, CH-4002 Basel, Switzerland

(*Received in Germany* 18 *March* 1991)

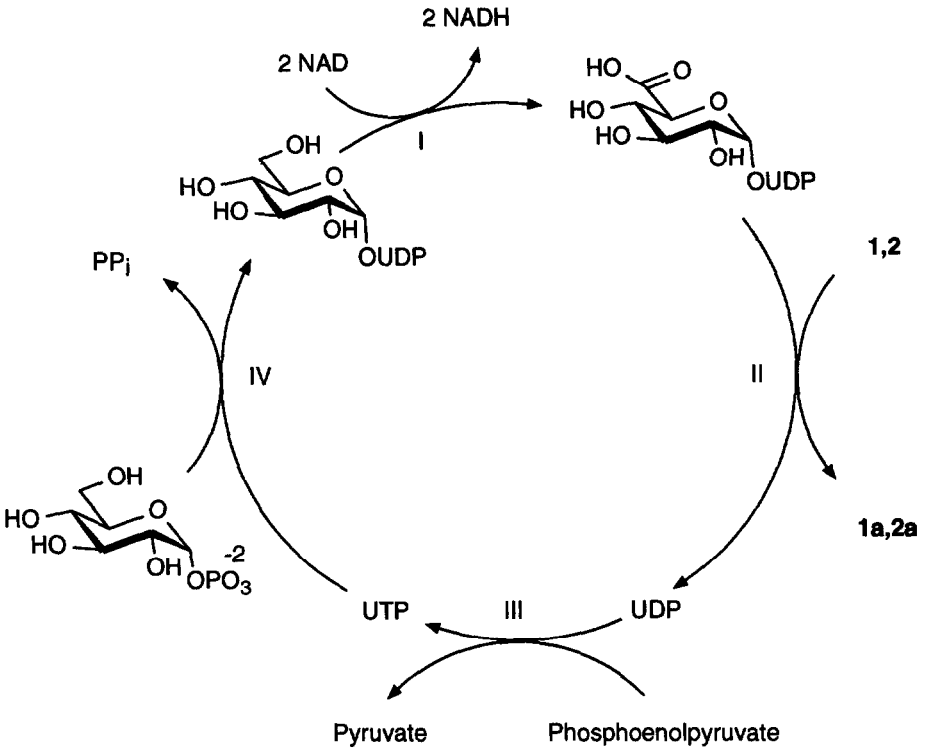
**Abstract:** β-D-glucuronides of **1** and **2** have been synthesized by a multi-enzyme system with *in situ* regeneration of uridine 5'-diphosphoglucuronic acid. Crude liver homogenate containing all enzymes involved in the multi-enzyme system was used for this stereoselective one-pot reaction.

β-D-Glucuronides of xeno- and endobiotics are widespread biotransformation products. They are required, both radioactively labelled or unlabelled in larger amounts for pharmacological, toxicological and analytical investigations.

The enzyme catalyzed synthesis of β-D-glucuronides is an alternative to chemical synthesis or isolation of these conjugates from biological materials. The UDP-glucuronyltransferases (EC 2.4.1.17) are a family of isoenzymes which catalyze the transfer of glucuronic acid from uridine 5'-diphospho-α-D-glucuronic acid to a suitable aglycon with inversion of the configuration at the anomeric centre of glucuronic acid.[1] So far this reaction has been used to conjugate aglycons such as phenols, amines, alcohols, thiols, carbamates and carboxylic acids. Glucuronides have been synthesized batch-wise or in a hollow fiber system using microsomal or soluble enzyme preparations.[2-4] Moreover they have been prepared with enzymes immobilized to polymeric supports.[5]

In this report we describe a simple one-pot reaction using glucose-1-phosphate as a donor of the glucuronic acid moiety and phosphoenolpyruvate and NAD as co-substrates. This multi-enzyme catalyzed glucuronidation enables the regeneration of uridine 5'-diphosphate sugars which are needed in catalytic amounts only (Scheme). A similar type of multi-catalyst system with immobilized enzymes has been used for the synthesis of oligosaccharides.[6,7] Instead of individually immobilized enzymes we used crude liver homogenate containing all enzymes involved in the multi-catalyst system.

The glucuronidation is started with catalytic amounts of UDP-glucose, which is oxidized by the NAD-dependent UDP-glucose dehydrogenase to UDP-glucuronic acid (I). Two products are generated during UDP-glucuronyltransferase-catalyzed transfer of glucuronic acid to the aglycon (II), namely the glucuronide




Scheme. Enzymatic glucuronidation with in situ regeneration of UDP-glucuronic acid.
I: UDP-glucose dehydrogenase, II: UDP-glucuronyltransferase, III: pyruvate kinase,
IV: UDP-glucose pyrophosphorylase. UDP: uridine 5'-diphosphate, NAD(H): β-nicotinamide adenine dinucleotide (reduced).

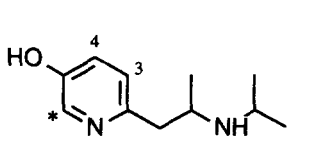
and UDP. The phosphorylation of UDP to UTP is catalyzed by pyruvate kinase (III) using phosphoenolpyruvate as phosphoryl-group donor. Finally, the cycle is closed by the UDP-glucose pyrophosphorylase-catalyzed transfer of UTP to glucose-1-phosphate (IV).

To demonstrate the feasibility of the enzymatic one-pot reaction we used two radioactively labelled compounds. Compound **1** is conjugated to a phenolic-O-glucuronide **1a** and the lipophilic steroid **2** to an enolic-O-glucuronide **2a**. The reaction was established and optimized by producing **1a** on an analytical scale. The formation of **1a** was followed by TLC and quantified by radioactivity scanning. Optimal conditions for the conjugation of 0.1 mmol of aglycon **1** were: 0.005 mmol of UDP-glucose, 0.6 mmol of glucose-1-phosphate, 0.4 mmol of phosphoenolpyruvate, 0.4 mmol of NAD and 100 mg protein of guinea-pig liver homogenate in 0.025 M of 4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid (HEPES) buffer (pH 8.0). In a preparative

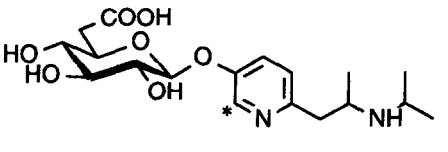
scale experiment, glucuronic acid was completely conjugated to **1** within 15 hours and yielded 45 mg (65%) of **1a**.

The lipophilic steroid **2** was added in solid state to the reaction mixture and consequently this reaction which was catalyzed best by crude rabbit liver homogenate was slow (7 hrs) and incomplete (about 30%) and yielded 2.7 mg (28%) of **2a**.

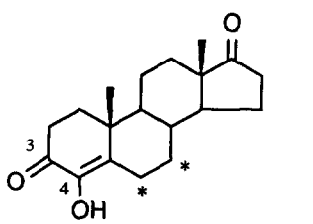
In summary, the multi-enzyme system with *in situ* regeneration of UDP-glucuronic acid provides an



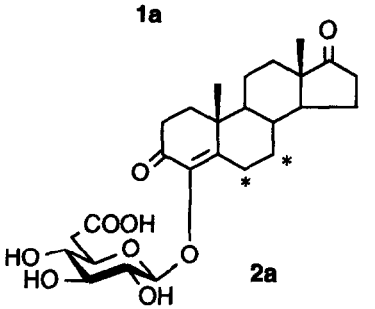

**1** * = $^{14}C$




**1a**




**2** * = $^{3}H$




**2a**

efficient route for the preparation of multi milligramm amounts of β-D-glucuronides. The value of this synthesis is that the costly and hydrolysis sensitive UDP-glucuronic acid is replaced by inexpensive glucose-1-phosphate, phosphoenolpyruvate and NAD. Instead of four individual enzymes a crude liver homogenate containing all the enzymes is used as multi-catalyst preparation. This preparation is sufficient for the synthesis of glucuronides. Finally, considering the broad aglycon acceptance of UDP-glucuronyltransferases this multi-enzyme catalyzed glucuronidation should also be applicable to other aglycons.

**Experimental Section**

**General.** FAB-MS spectra were recorded on a Finnigan MAT 90 mass spectrometer system equipped with an Ion Tech gun for fast atom bombardment (50 °C ion source temperature, thioglycerol matrix, 7 keV Xe fast atom beam). $^1$H-NMR spectra were recorded on a Varian Unity 500 NMR spectrometer, $D_2O$ or $CD_3OD$ solutions at room temperature. HPLC was performed either with a Spectra Physics SP 8100 pump using a Lichrosorb RP $C_{18}$ column (10 µm, 10 × 250 mm, Merck) with a Spectra Physics SP 8400 UV detector (270 nm) and radioactivity monitor LB 503, Berthold or Spectra Physics SP 8700 pump using a Partisil 5 ODS-3 column (4.6 × 250 mm, Whatman) with a Spectra Physics SP 8490 UV detector (275 nm) and a radioactivity monitor Ramona-D, Raytest, Burkhardt. The radioactivity was determined by a TLC radioactivity scanner (Automatic TLC-Linear-Analyzer Tracemaster 20, Berthold, Wildbad, Germany). Solvents for HPLC were purchased by Merck, Darmstadt, Germany. All reagents and substrates were purchased from Boehringer, Mannheim, Germany. Radioactively labelled **1** and **2** were provided by the Radiosynthesis Laboratory of CIBA-GEIGY Ltd., Basel.

**Preparation of crude liver homogenate used as multi-enzyme catalyst.** About 6 g of fresh liver from guinea pig or rabbit were homogenized (16'000 rpm, 0 °C, 1 min) in 19 ml 0.25 M glucose and centrifuged (4'500 g, 5 min). The supernatant which contains UDP-glucuronyltransferase activities was stored at -20 °C.

**Glucuronide formation.** Formation of glucuronide was followed by TLC (silica gel 60, F254 0.25 mm, Merck) with n-butanol-acetone-acetic acid-$NH_3$(25%)-$H_2O$ (70:50:18:1.5:60). $R_f$(**1a**)=0.36, $R_f$(**2a**)=0.65.

**Synthesis of 2-(2-isopropylaminopropyl)-5-pyridinol-β-D-glucopyranuronic acid (1a).** **1** (36.1 mg, 0.2 mmol, 84.1 kBq/mg), Glucose-1-phosphate disodium salt tetrahydrate (460.6 mg, 1.2 mmol), phosphoenol-pyruvate sodium salt (166.4 mg, 0.8 mmol), NAD (530.7 mg, 0.8 mmol), $MgCl_2$ hexahydrate (162.6 mg, 0.8 mmol), UDP-glucose disodium salt (6.1 mg, 0.01 mmol) were dissolved in 30 ml 0.1 M 4-(2-hydroxyethyl)-piperazine-1-propane-sulfonic acid (HEPPS) buffer, pH 8.0. The pH was adjusted to pH 8.0 by the addition of 2 M NaOH. HEPPS buffer and 4.0 ml of crude guinea pig liver homogenate (50 mg/ml protein) were added to a final volume of 40 ml. The brownish, turbid solution was stirred at 37°C for 15 h, at which time monitoring of the glucuronide formation, which was followed by TLC indicated 100% conversion. The reaction mixture was lyophilized and the residue dissolved in $H_2O$. The glucuronide **1a** was isolated and purified by HPLC on a Lichrosorb RP $C_{18}$ column. The glucuronide was separated in three subsequent isochratic HPLC steps at a flow rate of 2 ml/min. HPLC step 1: solvent A containing 20 mM ammonium acetate buffer pH 4.4 and solvent B containing acetonitrile (80% A/20% B, 0 to 30 min). HPLC step 2: 97.5% A/2.5% B (0 to 25 min). HPLC step 3: 98% A/2% B (0 to 30 min). Yield of **1a**: 45 mg (65%). FAB pos 371; $^1$H-NMR (500 MHz, $D_2O$): 8.33 (1H,d,pyridine H-6); 7.59 (1H,dd,pyridine H-4); 7.37 (1H,d,pyridine H-3); 5.15 (1H,m,$J_{1,2}$=7, glucuronyl H-1); 3.91 (1H,m,$J_{5,4}$=9,glucuronyl H-5); 3.80 (1H,sext,$CH_3\underline{CH}CH_2$); 3.58-3.68 (4H,m,glucuronyl H-2,H-3,& H-4,$CH_3\underline{CH}CH_3$); 3.21 & 3.01 (1H each,dd,$CH_2$); 1.35, 1.32 & 1.25 (3H each,d,$CH_3$). Position of glucuronidation ascertained by 1D NOE difference spectroscopy: Saturation of glucuronyl H-1 caused a strong NOE on pyridine H-4 and H 6.

**Synthesis of 4-Hydroxy-androst-4-ene-3,17-dione-β-D-glucopyranuronic acid (2a).** Glucose-1-phosphate disodium salt tetrahydrate (46.1 mg, 0.12 mmol), phosphoenolpyruvate sodium salt (16.64 mg, 0.08 mmol), NAD (53.07 mg, 0.08 mmol), $MgCl_2$ hexahydrate (16.26 mg, 0.08 mmol), UDP-glucose disodium salt (0.61 mg, 0.001 mmol) were dissolved in 40 ml 0.1 M HEPPS buffer, pH 8.0. **2** (6.04 mg, 0.02 mmol, 37.7 kBq/mg) was added in solid state, the pH was adjusted to pH 8.0. The reaction was started by the addition of 0.4 ml of crude rabbit liver homogenate (38 mg/ml protein). The reaction suspension was stirred at 37°C for 7 h, at which time monitoring of the glucuronide formation, which was followed by TLC indicated a maximum conversion of about 30%. The reaction mixture was lyophilized, the residue dissolved in $H_2O$ and **2a** was isolated and purified by RP $C_{18}$ HPLC chromatography. The separation was performed on a Partisil 5 ODS-3 column with 10 mM ammonium acetate buffer pH 5/methanol (55:45%, v/v) at a flow rate of 1 ml/min. Yield: 2.7 mg (28%). FAB pos 479; FAB neg 477; $^1$H-NMR (500 MHz, $CD_3OD$): 4.52 (1H,d,$J_{1,2}$=7.5, glucuronyl H-1); 3.51 (1H,d,$J_{5,4}$=9,glucuronyl H-5); 3.47 (1H,m,glucuronyl H-4); 3.45 (1H,m,H-6eq); 3.40 (1H,m,glucuronyl H-3); 3.37 (1H,m,glucuronyl H-2); 2.63 (1H,ddd,H-2ax); 2.45 (1H,dd,H-16); 2.39 (1H,dt,H-2eq); 1.96-2.15 (5H, H-1eq,H-6ax,H-7eq,H-15α,H-16); 1.68-1.83 (4H,H-1ax,H-8,H-11eq,H-12eq); 1.63 (1H,m,H-15β); 1.50 (1H,qd,H-11ax); 1.33 (1H,ddd,H-14); 1.27 (1H,td,H-12ax); 1.00-1.09 (2H,H-7ax,H-9). The assignments are based upon a COSY of **2** in $CD_3OD$ and the complete assignment of the $^1$H-NMR spectrum of testosterone.[8]

**Acknowlegdement.** We are grateful to Mrs. J. Burger and Mr. W. Gertsch for technical assistance.

**References**

1. Zakim D., Hochman Y., Vessey D.A.; Biochemical Pharmacology and Toxicology, **1985**, Zakim D. and Vessey D.A., Eds; John Wiley and Sons, New York, Vol. 1, pp 161-227.
2. Johnson Don M.J., Barker C.W., Fanska C.B., Murrill E.E.; Prep. Biochem. **1979**, 9, 391-406.
3. Guenthner T.M., Blair N.P.; Pharmacology **1988**, 37, 341-348.
4. Tegtmeier F., Belsner K., Brunner G.; Bioprocess Engineering **1988**, 3, 43-47.
5. Dulik D.M., Fenselau C.; FASEB J. **1988**, 2, 2235-2240.
6. Wong C.-H., Haynie S.L., Whitesides G.M.; J. Org. Chem. **1982**, 47, 5418-5420.
7. Augé C., David S., Mathieu C., Gautheron C.; Tetrahedron Lett. **1984**, 25, 1467-1470.
8. Hayamizu K., Ishii T., Yanagisawa M., Kamo O.; Magn. Reson. Chem. **1990**,28,250-256.