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Enzymatic Synthesis and Properties of Uridine-5'-*O*-(2-thiodiphosphoglucuronate)

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Abstract: Uridine-5'-*O*-(2-thiodiphosphoglucuronate) (UDP(βS)-GA) was synthesized in approximately 38% yield from UDP(βS)-glucose and nicotinamide adenine dinucleotide (NAD^+) in a reaction catalyzed by UDP-glucose dehydrogenase. UDP(βS)-GA was not a substrate for the *p*-nitrophenol glucuronosyltransferase of rat liver but was a better inhibitor of nucleotide phosphodiesterase than the natural compound.

Hepatic glucuronosyltransferases detoxify a wide variety of xenobiotic agents and endogenous metabolic products[1]. These enzymes can conjugate glucuronic acid to toxins, thereby rendering them more water-soluble and amenable to renal and biliary clearance. In certain cases of acute poisoning, such as acetaminophen overdose, the depletion of the hepatic pool of the cofactor, UDP-glucuronic acid (UDPGA) appears to be a rate limiting factor in clearance of the drug[2].

Intravenous administration of UDP-glucose, a metabolic precursor of UDPGA, provided a 25% reduction in toxicity to mice given subsequent lethal doses of acetaminophen[3]. It is likely that only a small amount of the injected UDP-glucose reached the intracellular sites of glucuronidation since this compound would be vulnerable to degradation by phosphodiesterases in serum and on liver cell surfaces[4,5]. We reasoned that administration of UDPGA might offer better protection from hepatic damage, provided it could escape degradation by tissue phosphodiesterases. Marchase and coworkers[6,7] have reported that β-phosphorothioate derivatives of UDP-glucose and UDP-galactose are one-tenth as sensitive to degradation by tissue phosphodiesterases as their normal counterparts while remaining reasonably good substrates for certain glycosyltransferases. Based on these observations, we sought to synthesize the β-phosphorothioate derivative of UDPGA (Figure 1) and to determine whether this analogue is a substrate for liver glucuronosyltransferase.

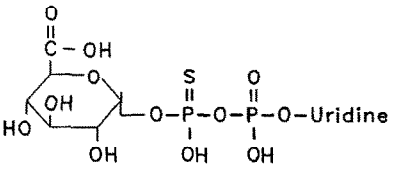

Figure 1. Structure of UDP(βS)-GA

Uridine-5'-*O*-(2-thiodiphosphoglucose) (UDP(βS)-glucose) was prepared according to the procedures of Singh et al.[8] with the exception that the reactions were catalyzed by immobilized enzymes[9]. UDP(βS)-glucose was purified

on DEAE-Sephadex A-25 with a linear gradient of 10-200 mM triethylammonium bicarbonate (TEAB), was freed of excess TEAB by repeated evaporations with water, and brought to a final concentration of 20 mM in 50 mM HEPES pH 7.5. Two milliliters each of 20 mM UDP(βS)-glucose, 100 mM NAD (in 50 mM HEPES, pH 7.5), and 0.5 M Tris acetate pH 8.5 were mixed with deionized water to a final volume of 20 ml and supplemented with two mls (approx. 2 units) of UDP-glucose dehydrogenase[10]. After incubation at 30°C for approximately 16 hours, the incubation mixture was cooled to 4°C, brought to a concentration of 10 mM TEAB, and applied at 25 ml/h to a column (1 x 13 cm) of DEAE-Sephadex A-25 pre-equilibrated with 10 mM TEAB. The column was washed successively with 1-2 bed volumes of 10 mM and 50 mM TEAB, followed by a linear 200 ml gradient of 50-600 mM TEAB, collecting 2.5 ml/fraction. Selected column fractions were monitored for absorbance at OD_{260}.

Peak fractions eluting near the end of the gradient were pooled, evaporated to dryness several times with water to remove excess TEAB, and redissolved in 5 ml of water. The concentration of UDP(βS)-GA in the pool was 3.1-3.2 mM (38-40% yield) as determined by three methods: absorbance at 260 nm, phenol-sulfuric acid assay for total carbohydrate[11], and by the carbazole assay for uronic acid[12]. FAB/MS in the negative mode yielded the expected anion peak at m/z 595. The absence of a peak at m/z 579 confirmed that the sample was free from contamination by UDPGA. Analysis of this material by thin layer chromatography on silica gel 60 in the solvent system 95% ethanol:0.1 M ammonium acetate, 2 mM EDTA, pH 7.0 7:3 (v/v) yielded a single spot (R_f 0.76) which was detected by either UV light or a starch/I_2/azide spray reagent specific for reactive sulfate groups[13]; the R_f of UDP(βS)-glucose in this system is 0.87. HPLC analysis of the UDP(βS)-GA was performed on a Partisil-10-SAX column (250 x 4.6 mm) using a phosphate gradient system[14] with a single peak eluting at approximately 46.5 minutes; UDPGA eluted at 44.3 minutes in the same system. The UDP(βS)-GA was converted from the TEAB to the sodium salt by passage through a column of AG50 (Na^+ form), then was lyophilized and redissolved in a small volume of 50 mM HEPES pH 7.5.

Liver microsomes were prepared[15] from adult male Sprague-Dawley rats which had been induced by two daily intraperitoneal injections of β-naphthoflavone (100 mg/kg). The microsomes were suspended in 0.25 M sucrose/10 mM Tris HCl, pH 7.4 to a final concentration of approximately 40 mg protein/ml and stored at -70°C until use. Glucuronosyltransferase activity, using p-nitrophenol as substrate at 0.5 mM, was assayed according to Bock et al.[16]. Under these conditions, we obtained an apparent K_m of 1.65 mM and V_{max} of 147 nmol/min/mg protein for UDPGA as a substrate. Under similar conditions, the β-phosphorothioate analogue was not a substrate for the liver glucuronosyltransferase even at the highest concentration tested, i.e., 10 mM. Although not a substrate for the transferase, UDP(βS)-GA did inhibit the reaction when UDPGA was used as substrate; when both UDPGA and UDP(βS)-GA were present at 3 mM, the product formed after a 30 minute incubation period was 80% of that formed with UDPGA alone.

Since UDPGA is a competitive inhibitor of nucleotide phosphodiesterase (PD) in liver membranes[17], we determined whether UDP(βS)-GA behaves similarly. PD activity was assayed using the method of Razzell[18] as modified by Watkins and Pierce[19]. UDP(βS)-GA and UDPGA are competitive inhibitors of the phosphodiesterase reaction (Figure 2). The calculated K_i's for UDP(βS)-GA and UDPGA were 0.006 and 0.045 mM, respectively.

Singh et al. previously reported[8] that UDP(βS)-glucose is an efficient substrate for UDP-glucose dehydrogenase as judged by the increase in absorbance at 340nm (reduction of NAD^+ to NADH). The presumed product of this reaction, UDP(βS)-GA, was neither isolated nor characterized. We have scaled up the reaction mixture and isolated and

characterized the phosphorothioate derivative of UDP-glucuronic acid. β-Phosphorothioate analogues of nucleotide sugars are efficient substrates for some but not all glycosyltransferase reactions. UDP(βS)-glucose is utilized nearly as well as the natural compound by the glycoprotein:glucose-1-phosphate glucosyltransferase of liver[6], but it is a very poor substrate for two other glucosyltransferases, sucrose synthetase and glycogen

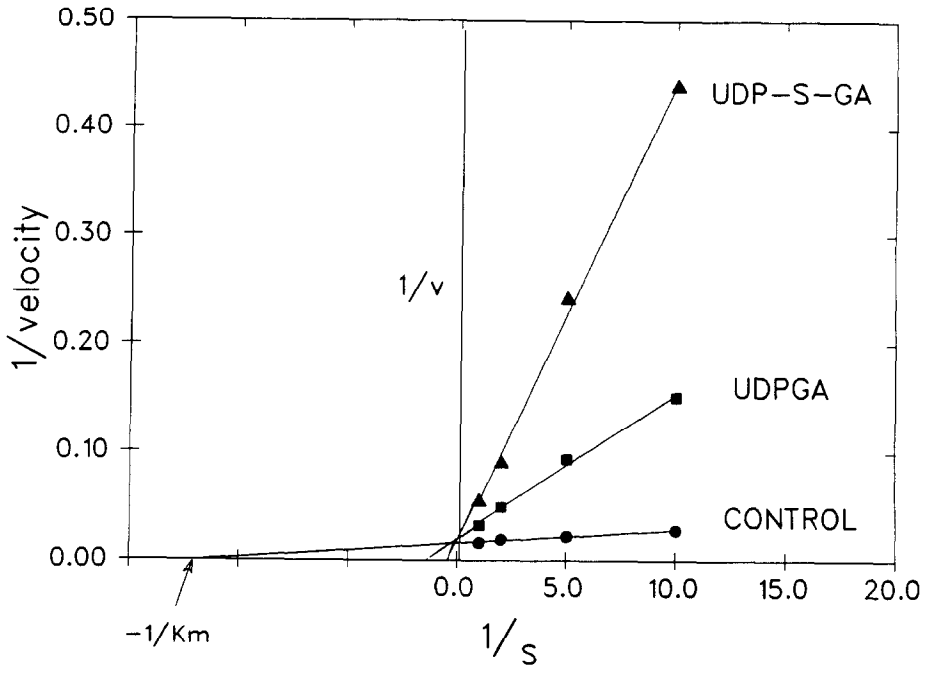

Figure 2. Competitive inhibition of liver phosphodiesterase activity by UDPGA and UDP(βS)-GA at a concentration of 0.3 mM.

synthetase[8]. We report here that enzymatically synthesized UDP(βS)-GA is not a substrate for the p-nitrophenyl glucuronosyltransferase of rat liver. Others[8] have suggested that the absolute configuration of the thiophosphoryl groups may be essential for recognition by the enzyme's active site. It is thus possible that the other diastereomer of UDP(βS)-GA may prove to be a substrate for the liver glucuronosyltransferase. It is also possible that the analogue reported here may prove to be an acceptable substrate for other UDPGA-utilizing enzymes, such as other classes of glucuronosyltransferases or UDP-glucuronic acid decarboxylase, which might have less stringent requirements for the phosphate-sugar linkage.

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