Enzymic synthesis of β-glucuronides of estradiol, ethynylestradiol and other phenolic substrates on a preparative scale employing UDP-glucuronyl transferase

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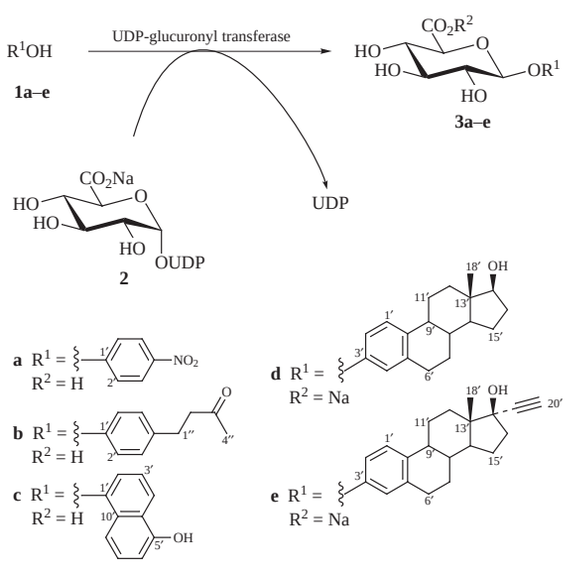
By incubation of bovine liver UDP-glucuronyl transferase with various phenolic aglycone substrates, preparation of the corresponding β-glucuronides could be achieved in milligram quantities. Concomitantly, simple access was generated to estrogen steroid glucuronides of great biological importance. In terms of experimental feasibility, direct application of the commercially available crude microsomal product was compared to and proven to be competitive with the usually preferred utilization of an immobilized enzyme preparation.

Introduction

Uridine 5′-diphospoglucuronyl transferases (EC 2.4.1.17) are a family of isoenzymes, ubiquitous in living organisms, which catalyze the synthesis of β-D-glucuronides from uridine 5′-diphospho-α-D-glucuronic acid (sodium salt: **2**) and a wide variety of endo- as well as xenobiotic aglycones.[1] The *in vitro* application of this important metabolic process is well documented on an analytical scale. However, in enzymic as opposed to classical chemical synthesis, extension of a successfully elaborated process from micro- to even milligram quantities often represents a far-from trivial task. With respect to glucuronyl transferases, there are only a few reports of the utilization of the enzymic reaction for preparative purposes.[2–6] A particular motivation for additional contributions to this area originates from the demand for glucuronic acid conjugates of many substances as widespread natural metabolites for biological and medical investigations. Chemical glucuronide synthesis frequently is accompanied by substantial difficulties due to the poor donor abilities of the respective glycosyl starting materials.[7] Among glycosyl transferases, the UDP-glucuronyl transferases are distinguished by their comparatively little sensitivity to incubation conditions and laboratory influences. This, as well as their broad tolerance of aglycone substrate structures, makes them appear as especially powerful tools for chemoenzymic synthesis.

Results and discussion

Since glucuronic acid transfer is reported to occur most efficiently to phenolic hydroxy groups,[5] our study focused on glycosylating a selection of phenolic substrates which are shown in Scheme 1. 4-Nitrophenol **1a** was considered especially suitable to serve as a model substrate for preparative glucuronylation as it is commonly employed to measure glucuronyl transferase activities.[8] In accordance with previous reports,[2,5] bovine liver UDP-glucuronyl transferase from the commercially available crude microsomal preparation was immobilized with Sepharose 4B by the CNBr method in order to facilitate work-up and product isolation as well as to enhance the thermostability of the enzyme. Based on this initial experiment (see Table 1) 4-nitrophenyl glucuronide **3a** could be



Scheme 1

Table 1 Preparation of glycosides **3a**–**e** from aglycones **1a**–**e** and UDP-glucuronic acid **2** with bovine liver UDP-glucuronyl transferase at 37 °C.

Aglycone	Enzyme preparation	Enzyme activity (units cm^{-3})	Cosolvent (% v/v)	Time (*t*/d)	Isolated yield [% (mg)]
1a	Sepharose-bound	0.015		3	25 (15) **3a**
1b	Sepharose-bound	0.030		5	
1b	Sepharose-bound	0.030	EtOH (1)	3	16 (14) **3b**
1c	Sepharose-bound	0.030	EtOH (1)	5	11 (7.5) **3c**
1d	Sepharose-bound	0.030	DMF (10)	5	5 (5) **3d**
1d	Crude microsomal	0.040	DMF (10)	3	34 (16) **3d**
1e	Sepharose-bound	0.030	DMF (10)	5	5 (5) **3e**
1e	Crude microsomal	0.040	DMF (10)	4	10 (5) **3e**

isolated in fairly good yield. After removal of the immobilized enzyme by centrifugation, the work-up procedure was much simplified by butan-1-ol extraction of the remaining aqueous solution, which resulted in an almost salt-free crude product fraction suitable for high-performance liquid chromatography (HPLC) purification directly after evaporation. Extension of the reaction to more complex aglycone substrates was attempted with doubled enzyme activity. The first attempt with 4-(3-oxobutyl)phenol **1b**, however, did not give the desired result. By addition of a small amount of ethanol as organic cosolvent, this problem could be solved, and both glucuronides **3b** and **3c** were obtained by the same convenient work-up procedure in, however, lower yields from the corresponding phenols **1b** and **1c**. Finally, the synthetic potential of the reaction could be elucidated by application to the steroidal sex hormone estradiol **1d** and its 17α-ethynyl derivative **1e**. Both compounds are of significant interest to diagnostic as well as therapeutic clinical medicine. Since they are *in vivo* predominantly metabolized as their glucuronic acid conjugates, the latter are largely required for a multitude of further investigations. The complex structure of the glucuronides **3d** and **3e**, in both of which an additional free aliphatic hydroxy group is present, renders them particularly interesting targets for enzymic synthesis. The poor solubility of these steroids in most aqueous and organic media required the addition of a substantial quantity of DMF in order to obtain the desired conversion. Preceding assays revealed that glucuronyl transferase activity was not affected by having DMF as cosolvent. Nevertheless, isolated yields of glucuronides **3d** and **3e** were comparatively poor. A reason for this can be seen in the modified work-up procedure, which was required since the crude steroid glucuronides could not be enriched by solvent extraction. Thus, a major problem arose from the large amount of buffer salts present which necessitated repeated chromatographic purification steps. In order to shorten the overall process of preparation, an attempt was made to omit enzyme immobilization and achieve the same synthesis by direct application of the commercial microsomal preparation. Therefore, the initially applied activity was slightly increased to overcome for a possible degradation of the transferase in this less stable state. Under these conditions, shorter incubation periods resulted in isolated yields that were comparable or even superior to those obtained before. Thus, the simple direct employment of a commercially available crude enzyme preparation was demonstrated to be a considerable alternative to the hitherto often preferred immobilization strategy.

In conclusion, we rate the results of the presented synthetic studies as encouraging for the further elucidation of enzymic glucuronylation as a potent preparative method to obtain complex glucuronic acid conjugates, which might not be easily accessible by classical chemical means.

Experimental

General

Bovine liver UDP-glucuronyl transferase (EC 2.4.1.17) was purchased from Sigma as a crude microsomal preparation with an activity of 2.2 units g^{-1} solid (Sigma U 4502) (one unit will transfer 1.0 μmol of glucuronic acid from UDP-glucuronic acid to phenolpnthalcin per min at pH 8.0 at 37 °C). For immobilization, commercial CNBr-activated Sepharose 4B (Sigma) was employed. TLC was performed on silica 60-coated aluminium sheets (Merck or Macherey-Nagel) using the following eluent mixture: butan-1-ol–water–acetone–glacial acetic acid–25% aq. ammonia (70 : 60 : 50 : 18 : 1.5). Spots were visualized under UV light at 254 nm and by spraying with 0.2% naphthoresorcinol in acetone–10% (ortho)phosphorous acid in water (5 : 1 v/v) and subsequent heating. NMR spectra were recorded on a Bruker AMX-400 NMR spectrometer. *J*-Values are given in Hz. Optical rotations were measured with a Perkin-Elmer polarimeter 243 or 341, with $[\alpha]_D$-values given in units of 10^{-1} deg cm^2 g^{-1}.

Immobilization procedure

All steps were carried out at temperatures between 0 and 4 °C. CNBr-activated sepharose (2 g) was soaked in 5 mM hydrochloric acid (7 cm^3) for 15 min. The resulting gel was washed with 5 mM hydrochloric acid (400 cm^3) and coupling buffer [0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3 (3 × 20 cm^3)] and added to a solution of UDP-glucuronyl transferase preparation (125 mg) in coupling buffer (10 cm^3). This suspension was shaken for 24 h, and the immobilizate was separated by centrifugation, washed with coupling buffer (3 × 20 cm^3), suspended in blocking buffer [0.5 M NaCl, 0.1 M Tris-HCl, pH 8.0 (10 cm^3)], and shaken for another 24 h. The thus obtained preparation contained an approximate transferase activity of 0.3 units and was used immediately or transferred into preserving buffer [50 mM Tris-HCl, 0.1% (w/v) sodium azide, pH 8.0] and stored for several weeks at 0 °C. In each case, the gel was washed with incubation buffer [0.3 M Tris-HCl, 1 mM calcium chloride dihydrate, pH 8.0 (3 × 25 cm^3)] prior to use.

Enzymic glycoside synthesis: procedure A

Amounts of incubation buffer and organic cosolvent were calculated to add up to a total volume of 10 cm^3. UDP-glucuronic acid (trisodium salt **2**; 129 mg, 0.2 mmol) and the aglycone (0.2 mmol) as a solution in the respective cosolvent or as pure solid were added to a suspension of sepharose-bound UDP-glucuronyl transferase (activity as specified in Table 1) in incubation buffer. The mixture was gently shaken at 37 °C for the period specified in Table 1 and then separated by centrifugation or filtration. Further work-up and product isolation procedures are described below.

Enzymic glycoside synthesis: procedure B

UDP-glucuronic acid (trisodium salt **2**; 65 mg, 0.1 mmol) and a solution of the aglycone (0.1 mmol) in DMF (0.5 cm^3) were added to a suspension of the crude microsomal preparation of UDP-glucuronyl transferase (0.2 units) in incubation buffer (4.5 cm^3). The mixture was gently shaken at 37 °C for the period specified in Table 1 and then separated by centrifugation. After extraction of the solid residue with water (3 × 20 cm^3), the combined aqueous solutions were freeze-dried, and the residual crude product was purified by column chromatography on Biogel P2.

4-Nitrophenyl β-D-glucopyranosiduronic acid 3a

Procedure A was applied to 4-nitrophenol **1a**. For work-up, the combined aqueous solutions were acidified with 5 M hydrochloric acid to pH 3.5, washed with diethyl ether (3 × 70 cm^3), and extracted with butan-1-ol (3 × 70 cm^3). The combined butanol extracts were evaporated under reduced pressure at 20 °C and the residual crude product was purified by reversed-phase HPLC to give title compound **3a** as a solid, $[\alpha]_D^{20}$ −41.2 (*c* 0.7, water) {lit.,[9] $[\alpha]_D^{17}$ −108 (*c* 1, EtOH)}; δ_H (400 MHz; D_2O) 3.63 (2 H, dd, $J_{2,3}$ 8.6, $J_{3,4}$ 7.3, 3- and 4-H), 3.67 (1 H, m, 2-H), 3.97 (1 H, d, $J_{4,5}$ 8.6, 5-H), 5.28 (1 H, d, $J_{1,2}$ 7.0, 1-H), 7.25 (2 H, d, *J* 9.2, 3′- and 5′-H) and 8.26 (2 H, d, *J* 9.2, 2′- and 6′-H); δ_C (100.6 MHz; D_2O) 74.50 (C-4), 75.48 (C-3), 78.10 (C-2), 79.13 (C-5), 102.24 (C-1), 119.43 (C-2′ and -6′), 128.96 (C-3′ and -5′), 145.47 (C-4′), 164.72 (C-1′) and 175.64 (C-6).

4-(3-Oxobutyl)phenyl β-D-glucopyranosiduronic acid 3b

Procedure A was applied to 4-(3-oxobutyl)phenol **1b**. For work-up, the combined aqueous solutions were acidified to pH 4 with 5 M hydrochloric acid and further treated as described for the preparation of compound **3a** to give title compound **3b** as a solid, $[\alpha]_D^{20}$ −44.2 (*c* 0.7, water); δ_H (400 MHz; D_2O) 2.20 (3 H, s, 4″-H), 2.87 (4 H, m, 1″- and 2″-H), 3.58 and 3.62 (3 H, m, 2-, 3- and 4-H), 3.87 (1 H, d, $J_{4,5}$ 8.9, 5-H), 5.08 (1 H, d, $J_{1,2}$ 7.0,

1-H), 7.09 (2 H, d, *J* 8.5, 2′- and 6′-H) and 7.24 (2 H, d, *J* 8.5, 3′- and 5′-H); δ_C (100.6 MHz; D_2O) 29.40 (C-4″), 30.60 (C-2″), 45.49 (C-1″), 72.72, 73.80 and 76.35 (C-2, -3 and -4), 77.10 (C-5), 101.38 (C-1), 117.86 (C-2′ and -6′), 130.52 (C-3′ and -5′), 136.50 (C-4′), 155.70 (C-1′), 174.00 (C-6) and 216.16 (C-3″).

5-Hydroxynaphthalen-1-yl β-D-glucopyranosiduronic acid 3c

Procedure A was applied to 1,5-dihydroxynaphthalene **1c**. For work-up, the combined aqueous solutions were freeze-dried, the residual solid was dissolved in water (2 cm^3), and the solution was extracted with butan-1-ol (3 × 20 cm^3). After evaporation of the combined organic extracts under reduced pressure, the crude product was purified by ion-exchange HPLC to give title compound **3c** as a solid; δ_H (400 MHz; D_2O) 3.68 (2 H, m, 3- and 4-H), 3.82 (1 H, dd, $J_{1,2}$ 8.0, $J_{2,3}$ 8.0, 2-H), 3.95 (1 H, d, $J_{4,5}$ 9.5, 5-H), 5.30 (1 H, d, $J_{1,2}$ 8.0, 1-H), 7.00, 7.29, 7.86 and 7.93 (4 × 1 H, 4 d, *J* 8.0, 2′-, 4′-, 7′- and 9′-H) and 7.46 and 7.49 (2 × 1 H, 2 dd, *J* 8.0, 3′- and 8′-H).

Sodium [17β-hydroxyestra-1,3,5(10)-trien-3-yl β-D-glucopyranosid]uronate[10] 3d

Procedures A and B were applied to 3,17β-dihydroxyestra-1,3,5(10)-triene (17β-estradiol) **1d**. For work-up after procedure A, the combined aqueous solutions were freeze-dried and the residual crude product was purified by column chromatography on Biogel P2 to give title compound **3d** as a solid, $[\alpha]_D^{20}$ −3.9 (*c* 0.5, water) {lit.,[10] mp 256–260 °C}; δ_H (400 MHz; D_2O) 0.53 (3 H, s, 18′-H_3), 0.91–1.28, 1.49, 1.68, 1.89, 2.08 and 2.62 (7 H, 1 H, 2 × 2 H, 1 H and 2 H, m), 3.41–3.45 (3 H, m, 2-, 3- and 4-H), 3.51 (1 H, d, 17′-H), 3.67 (1 H, d, $J_{4,5}$ 9.2, 5-H), 4.85 (1 H, d, $J_{1,2}$ 7.1, 1-H), 6.71 (1 H, d, ArH), 6.75 (1 H, dd, 2′-H) and 7.11 (1 H, d, ArH); δ_C (100.6 MHz; D_2O) 11.04 (C-18′), 22.90, 26.19, 26.92, 29.43 and 36.55 (C-6′, -7′, -11′, -12′, -15′ and -16′), 38.62, 43.75 and 49.67 (C-8′, -9′ and -14′), 43.07 (C-13′), 72.14, 73.18 and 75.76 (C-2, -3, -4 and -5), 81.79 (C-17′), 100.88 (C-1), 114.58, 117.19 and 127.19 (C-1′, -2′ and -4′), 136.15 and 139.43 (C-5′ and -10′) and 154.97 (C-3′).

Sodium [17α-ethynyl-17β-hydroxyestra-1,3,5(10)-trien-3-yl β-D-glucopyranosid]uronate[11,12] 3e

Procedures A and B were applied to 3,17β-dihydroxy-17α-ethynylestra-1,3,5(10)-triene (lynestrenol) **1e**. Work-up after procedure A was carried out as described for the preparation of compound **3d** to give title compound **3e** as a solid, $[\alpha]_D^{20}$ −1.3 (*c* 0.1, water); δ_H (400 MHz; D_2O) 0.66 (3 H, s, 18′-H_3), 1.06, 1.24, 1.46–1.71, 1.82, 1.95, 2.12 and 2.64 (1 H, 3 H, 5 H, 2 × 1 H and 2 × 2 H, m), 3.39–3.45 (4 H, m, 2-, 3-, 4- and 20′-H), 3.69 (1 H, d, $J_{4,5}$ 9.2, 5-H), 4.87 (1 H, d, $J_{1,2}$ 7.1, 1-H), 6.71 (1 H, d, ArH), 6.77 (1 H, dd, 2′-H) and 7.14 (1 H, d, ArH); δ_C (100.6 MHz; D_2O) 12.64 (C-18′), 22.59, 26.38, 26.98, 29.49, 33.06 and 38.63 (C-6′, -7′, -11′, -12′, -15′ and -16′), 39.14, 43.54 and 49.47 (C-8′, -9′ and -14′), 47.14 (C-13′), 72.14, 73.18 and 75.76 (C-2, -3, -4 and -5), 80.18 (C-17′), 101.05 (C-1), 114.63, 117.20 and 127.22 (C-1′, -2′ and -4′), 136.04 and 139.60 (C-5′ and -10′) and 155.20 (C-3′).

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