ENZYMATIC PREPARATION OF LYSERGOL β-D-GLUCURONIDE

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Enzymatic synthesis of lysergol β-D-glucuronide (**2**) using ovine microsomal uridine 5′-diphosphoglucuronyl transferase and uridine 5′-diphosphoglucuronic acid (Na_3-salt) is demonstrated.

Key words: Ergot alkaloids; Lysergol; β-D-Glucuronide; Conjugation; Glycosidation; Biocatalysis.

Ergot alkaloids (EA) are widely used in many fields of medicine. At present, there exist more than 50 formulations in clinical use containing natural or semisynthetic ergot alkaloids[1]. EA are produced by filamentous fungi from genus *Claviceps* (*e.g.*, *Claviceps purpurea* – Ergot, Mutterkorn) and by some other filamentous fungi and can also be found infrequently in the plant kingdom.

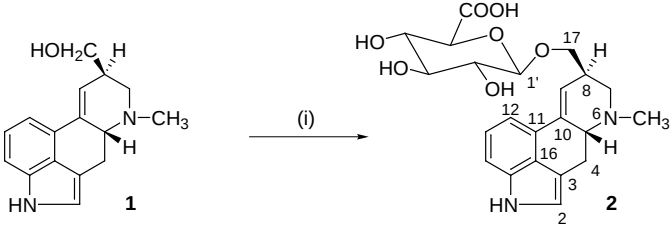
Ergot is, however, a two edged sword. Ergot alkaloids may contaminate human and animal food, either from ergot grains occurring in the corn or as the products of endophytic fungi in the pasture grass. This relatively new type of ergot toxicosis was first described as “fescue toxicosis”, but other pasture grasses can be affected, too. It is caused by endophytic fungi of the genus *Neotyphodium* (formerly *Acremonium*) which are systemic symbionts in several important genera of pasture grasses, including *Festuca* and *Lolium*[2].

Metabolites of EA are, therefore, important not only from the point of toxicology, pharmacokinetics and forensic chemistry (*e.g.*, LSD) but also in agriculture. EA are metabolized in mammals in many different ways depending on the respective alkaloid. Metabolism and biotransformations of natural and semisynthetic ergot alkaloids were recently reviewed by Křen[3]. So far, mostly oxidative and degradative products of EA have been isolated

and identified. Although conjugation (glucuronidation, sulfatation) of EA is known, such conjugates of EA have never been isolated because the metabolites were usually treated with hydrolytic enzymes (glucuronidase/sulfatase from *Helix pomatia*) and only released aglycons were eventually isolated.

A large series of various glycosides of EA was recently prepared by chemical and enzymatic methods[4]; however, preparation of β-glucuronides of any ergot alkaloid has not previously been achieved. Lysergol β-D-glucuronide (**2**) was chosen as a target compound because of the need of an authentic sample for MS and ELISA analyses of forage and animal material contaminated by ergot alkaloids from infected grass. For such studies only miligram(s) amount of the compound is needed. Lysergol (**1**) is produced by the *Claviceps* fungi parasiting on the grass *Agropyrum semicostatum*[5a] and it is also produced (besides ergopeptide alkaloid ergovaline) by endophytic *Neotyphodium* fungi[5b].

Here we describe the enzymatic preparation of the β-D-glucuronide of lysergol which, besides its biological significance, served also as a model ergot alkaloid for the enzymatic glucuronidation.



(i) Uridine 5'-diphosphoglucuronic acid, 5'-diphosphoglucuronyl transferase

EXPERIMENTAL

^{1}H and ^{13}C NMR spectra were measured on a Varian INOVA-400 spectrometer (399.91 and 100.57 MHz, respectively) in D_2O at 30 °C. Chemical shifts are given in the δ-scale, *J*-values in Hz. Carbon signal multiplicity was determined by an Attached Proton Test (APT) experiment. Manufacturer's software was used for 2D NMR (COSY, HOM2DJ, ROESY, HMQC, HMBC).

Positive-ion electrospray ionization (ESI) mass spectrum was recorded on a double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, Germany) with BE geometry. A sample dissolved in MeOH–H_2O 1 : 1 (v/v) was continuously infused through a stainless capillary held at 3.3 kV into the Finnigan ESI source *via* linear syringe pump at a flow rate of 20 μl min^{-1}. For high-resolution experiments the instrument was tuned to a resolution of about 7 000 (10% valley definition) and the measurements were carried out by the

peak-matching method using a mixture of poly(propylene glycol)s (average MW 425) as an internal standard.

Materials. Ovine liver microsomes, containing uridine 5′-diphosphoglucuronyl transferase (UDPGT) were prepared as described previously[6]. Uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA, uridine 5′-(α-D-glucopyranurosyl diphosphate)) was from Boehringer Mannheim (Mannheim, Germany). Buffers, solvents and general chemicals were obtained from local suppliers. LiChroprep RP-18 flash silica gel (particle size 25–40 μm) and aluminium-backed silica gel $60F_{254}$ TLC plates were obtained from Merck (Darmstadt, Germany). Lysergol (**1**), was a kind gift of Galena Pharmaceuticals Co., Ltd., (Opava, Czech Republic).

Small-Scale Lysergol β-D-Glucuronide (**2**) Synthesis (0.2 ml)

Reaction conditions were based on those developed for benzyl alcohol and 4-methyl phenol[7]. The substrate **1** in DMSO (160 mM stock solution, 10 μl for 8 mM final concentration) was added to the Tris-buffer (200 μl, 200 mM, pH 8.0) containing $CaCl_2$ (6 mM), UDPGA (16 mM), dithiothreitol (1 mM), BSA (3% (w/v)), and microsomal UDPGT (4 mg). The reaction was carried out in an Eppendorf tube at 30 °C and terminated after 24 h by addition of acetonitrile (200 μl), precipitated protein removed by centrifugation at 13 000 rpm for 10 min in a microfuge (Micro Centaur, MSE, U.K.), and the supernatant assayed for **1** and **2** by gradient HPLC.

Gradient HPLC. Supernatant (20 μl) was analyzed by HPLC on a Nucleosil 5μ, 100Å, C18 column (250 × 4.6 mm) (Phenomenex, Torrance (CA), U.S.A.) fitted to a Gilson (Middleton (WI), U.S.A.) dual pump HPLC system equipped with an autoinjector and a UV-visible diode array detector (Gilson). Elution was at 0.7 ml min^{-1} with a gradient of 0–40% acetonitrile in 0.01 M phosphoric acid. The eluted compounds were detected by UV absorbance at 230 nm. Under these conditions the retention times of **1** and **2** were 13.85 and 12.38 min, respectively.

Preparative HPLC. Preparative HPLC was performed on a Waters 600 system equipped with a Waters 486 UV detector and fitted with a Zorbax ODS column (21.2 mm × 25 cm).

Preparative-Scale Lysergol β-D-Glucuronide (**2**) Synthesis

The analytical-scale reaction was scaled up to a reaction volume of 27 ml (in a stoppered conical flask, stirred magnetically and purged with argon), with substrate (**1**; 55 mg) and other conditions identical. After 24 h, EDTA (100 mg, 0.27 mmol) was added to the reaction mixture (to give a concentration of 10 mM) to prevent formation of intractable calcium salts of the glucuronides, and the pH adjusted to 3.5 with 0.1 M HCl. The reaction mixture was diluted with three volumes of acetonitrile and the resulting proteinaceous precipitate removed by filtration through a filter aid. The precipitate was resuspended in water, reprecipitated with three volumes of acetonitrile, and refiltered. The filtrates were combined and the solvent removed *in vacuo*. The residue was dissolved in 0.1 M KCl (100 ml), filtered, and applied to a column (35 × 1.25 cm) of reversed-phase silica gel (LiChroprep RP-18). The column was eluted with water (100 ml) followed by a gradient of 0–40% acetonitrile (total volume 400 ml). Fractions (8 ml) were collected and pooled as indicated by TLC (elution with ethyl acetate–methanol–acetic acid 67 : 30 : 3), and solvent removed *in vacuo*. The crude product was further purified by preparative HPLC.

Lysergol β-D-*glucuronide* (**2**) ^{1}H NMR (399.91 MHz, D_2O, 30 °C): 2.858 m, 1 H (H-4a); 3.011 s, 3 H (N^+-Me); 3.070 dd, 1 H, *J*(7a,7e) = 11.3, *J*(7a,8) = 11.3 (H-7a); 3.180 m, 1 H (H-8); 3.359 dd, 1 H, *J*(1′,2′) = 7.9, *J*(2′,3′) = 9.5 (H-2′); 3.536 dd, 1 H, *J*(2′,3′) = 9.5, *J*(3′,4′) = 11.0 (H-3′); 3.598 dd, 1 H, *J*(3′,4′) = 11.0, *J*(4′,5′) = 9.2 (H-4′); 3.650 m, 1 H (H-7e); 3.747 dd, 1 H, *J*(17d,17u) = 10.6, *J*(8,17u) = 5.0 (H-17u); 3.767 dd, 1 H, *J*(4a,4e) = 14.6, *J*(4e,5) = 4.4 (H-4e); 3.941 m, 1 H (H-5); 4.072 dd, 1 H, *J*(17d,17u) = 10.6, *J*(8,17d) = 5.4 (H-17d); 4.161 d, 1 H, *J*(4′,5′) = 9.2 (H-5′); 4.517 d, 1 H, *J*(1′,2′) = 7.9 (H-1′); 6.548 br s, 1 H (H-9); 7.178 d, 1 H, *J*(2,4a) = 1.8 (H-2); 7.278 m, 1 H (H-13); 7.295 m, 1 H (H-12); 7.405 m, 1 H (H-14). ^{13}C NMR (100.56 MHz, D_2O, 30 °C): 27.69 t (C-4); 37.48 d (C-8); 44.43 q (N-Me); 58.10 t (C-7); 63.35 d (C-5); 74.20 t (C-17); 74.69 d (C-3′); 75.80 d (C-2′); 78.98 d (C-4′); 87.08 d (C-5′); 105.57 d (C-1′); 110.60 s (C-3); 113.62 d (C-14); 114.90 d (C-12); 123.08 d (C-2); 124.12 d (C-9); 126.17 d (C-13); 129.13 s (C-16); 135.59 s (C-15); 136.67 s (C-11); 140.79 s (C-10); 178.57 s (C-6′). HR ESI MS (**2**), *m/z*: 475.1465 $[M + Na]^+$ (475.1457 calculated for $C_{22}H_{25}N_2Na_2O_7$).

RESULTS AND DISCUSSION

Small-scale (0.2 ml) enzymic glucuronidation gave a yield of 23%, determined by HPLC. The preparative reaction, however, for unknown reasons, gave a yield of only 8% from a 27-ml reaction. Yields of glucuronidation using UDPGT are generaly quite high especially with phenolic compounds[7,8]. In case of ergot alkaloids the yields could be lower because of "Zwitterion" character of the product which causes also problems in separation. Chromatography on RP-18 did not separate **1** and **2** from each other but did free them from salts, DMSO and other water-soluble material from the reaction mixture. Final purification was achieved by preparative HPLC.

ESI mass spectrum of compound **2** contains two prominent peaks at *m/z* 475.1 and 491.1 corresponding to $[M + Na]^+$ and $[M + K]^+$ adduct ions of lysergol β-D-glucuronide sodium salt, respectively. The formation of the expected glucuronide was further confirmed by high-resolution mass spectrometry.

NMR data of **2** agree with the proposed structure. The sugar configuration is β (*J*(1′,2′) = 7.9 Hz), it is attached at C-17 (downfield shift of this carbon with respect to parent compound **1**); D ring nitrogen is protonated (downfield shift of the N-Me singlet). The reported assignment is based on COSY, TOCSY, HOM2DJ, and HMQC experiments. Many alkaloid signals are broad so that the extraction of some couplings was not possible; strong coupling was the reason in the case of the A ring protons.

This method using glucuronyl transferase seems to be very suitable for the preparation of the β-glucuronides of ergot alkaloids. Other attempts, *e.g.*, chemical glycosylations are not practicable due to harsh reaction conditions (aglycone destruction) and also due to production of ortho esters[4a]. Reverse glycosylation of some ergot alkaloids using β-glucuronidases (*E.*

coli, *Helix pomatia*) and 4-nitrophenyl β-D-glucuronide as a donor was tested, however, the yields of the glucuronides were poor[9]. Therefore, the method using glucuronyltransferase seems to be the method of choice for the preparation of the conjugates of ergot alkaloids although the yields could be still improved.

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