Preparative Enzymatic Synthesis of the Acylglucuronide of Mycophenolic Acid

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Abstract: The acylglucuronide (**3**) of mycophenolic acid (**1**) was enzymatically synthesised on a preparative scale (450 mg substrate) under optimised reaction conditions with 51% conversion. By screening 9 liver homogenates from 8 vertebrate species, it was shown that only with liver homogenate from horse as the catalyst were the acyl- (**3**) and the *O*-glucuronide (**2**) were formed in a *ca.* 1:1 ratio. With homogenates from other sources, the *O*-glucuronide **(2)** was produced in high excess. By optimising the concentration of the co-substrate UDP-glucuronic acid and the reaction temperature, the conversion to the acylglucuronide **(3)** was increased from initially 34 to 55% and the ratio of acyl- (**3**) to *O*-glucuronide (**2**) from 1.5:1 to 3.9:1. The reaction was also performed continuously in an enzyme membrane reactor, however, with lower conversion yield and therefore, higher specific UDP-glucuronic acid consumption.

Keywords: biotransformations; enzymatic synthesis; glucuronidation; metabolism; mycophenolic acid; transferases

Introduction

Mycophenolic acid (**1**) was first isolated from *Penicillium* sp. in 1896.[1] Its antibacterial and antifungal properties were detected in 1946[2,3] and its antiviral and antitumour activities in 1968.[4] Mycophenolate mofetil (CellCept®, Roche Inc., Basel, Switzerland) was developed in the 1960's as an antibiotic, antineoplastic and antipsoriatic drug, and was later also used for immunosuppression after solid tumour transplantations.[5] The sodium salt of mycophenolic acid **(1)** is currently being launched as an immunosuppressive drug for transplantation in the form of a new gastric juice-resistant formulation (Myfortic®, Novartis Inc., Basel, Switzerland) with fewer side effects. In man the major metabolic pathway of **1** is the phenolic glucuronidation to *O*-glucuronide **2** (see Figure 1) leading to a loss of pharmacological activity.[6] The acylglucuronide (**3**) of mycophenolic acid (**1**) has been identified as a pharmacologically active metabolite inhibiting the proliferation of mononuclear leukocytes[7] *via* inhibition of inosine monophosphate dehydrogenase II with similar efficiency as mycophenolic acid itself.[8] Both glucuronides are presumably formed by the same glucuronosyltransferase isoform (UGT1A10) in man.[9]

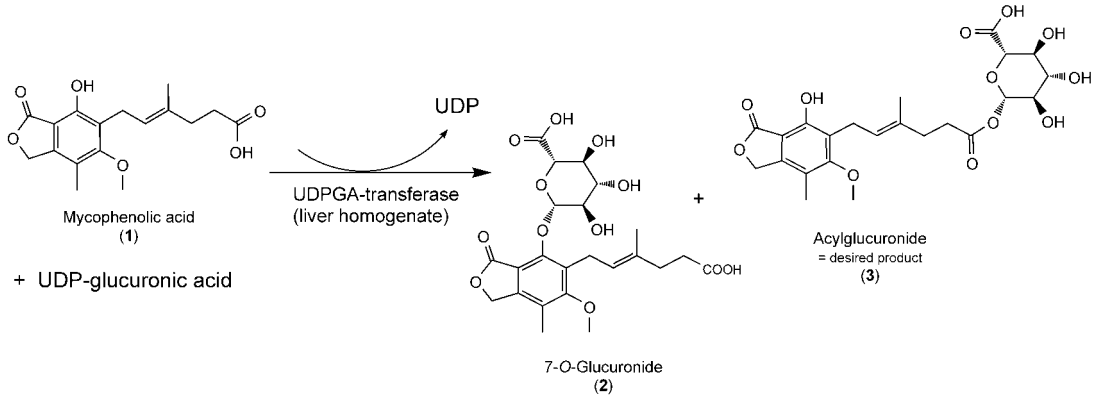

Figure 1. Enzymatic glucuronidation of mycophenolic acid.

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Table 1. Screening of liver homogenates from 8 vertebrate species for the formation of the acylglucuronide (**3**) of mycophenolic acid (**1**) [mycophenolic acid (**1**) 5 mM, UDPGA 40 mM, pH 7, 37 °C, 20% liver homogenate].

Liver homogenate from	pH 7		pH 8	
	Conversion [%] to		Conversion [%] to	
	Acylglucuronide	*O*-Glucuronide	Acylglucuronide	*O*-Glucuronide
Sheep	0	88	n.d.	n.d.
Pig	0	91	0	54
Rabbit	0	98	0	91
Cow	2	25	1.7	19
Chicken	4.3	44	1.3	41
Rat	6.5	67	1.9	41
Dog	9.9	81	8.4	55
Foal	27	41	16	17
Horse	24	28	12	11

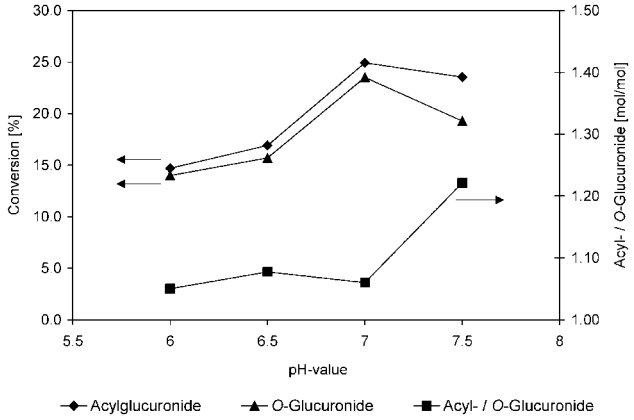

Figure 2. Glucuronidation of mycophenolic (**1**) acid with horse liver homogenate at different pH values (40 mM UDPGA, 37 °C, 20% liver homogenate).

Results and Discussion

The acylglucuronide was requested for pharmacological studies by the transplantation department within Novartis Institutes of Biomedical Research. Since in human metabolism the ratio of acyl- to 7-*O*-glucuronide is 1 : 80,[10] the preparative scale synthesis of the acylglucuronide (**3**) using human liver microsomes as the catalyst appeared to be impracticable.

Therefore, in the beginning of the investigations we screened our biocatalyst library for glucuronidation consisting of 9 liver homogenates from 8 vertebrate species. As can be seen in Table 1 both the acyl- and the *O*-glucuronide are produced with much higher efficiency at pH 7 than at pH 8. Also with human liver microsomes, mycophenolic acid glucuronidation is favoured at pH 7.[5] Only when applying the liver homogenates from foal or horse was it possible to obtain the acylglucuronide of mycophenolic acid in significant quantities. With horse liver homogenate the ratio of acyl- to *O*-glucuronide was even close to one, so that, in regard to product purification, it was chosen as the enzyme source. Similarly, the *O*-glucuronidation of Sch 60664, a structural analogue of a cholesterol absorption inhibitor, is described to be only possible under catalysis of dog liver microsomes, but not with those from rat and monkey liver or rabbit intestine.[11]

From the literature it is known that the optimum pH for the glucuronidation of different substrates catalysed by human liver microsomes can vary considerably.[12] When the pH of the reaction with 20% horse liver homogenate was varied, at pH values below 7 a drop of glucuronidation activity was observed for both products (see Figure 2). Since the results for pH 7 and 7.5 were quite similar and since acylglucuronides are generally more stable in the acidic range,[13] pH 7 was chosen for the further experiments.

UDP-glucuronic acid (UDPGA) is the most expensive ingredient used in the enzymatic glucuronidation reactions catalysed by UDPGA-glucuronosyl transferases. Therefore, we investigated the influence of the UDPGA concentration on the glucuronidation of mycophenolic acid (see Figure 3). The ratio of acyl- to *O*-glucuronide formation increased continuously with decreasing UDPGA concentration. At 15 mM starting concentration of the co-substrate the specific consumption of UDPGA showed a minimum of 18 moles per mol of acylglucuronide formed. Thereby, *ca.* 32% of the maximum conversion to the acylglucuronide (which was obtained with 60 mM UDPGA, see Figure 3) was sacrificed. 25 mM UDPGA was considered as a reasonable compromise between co-substrate consumption and final acylglucuronide concentration (only 16% loss compared to the maximum conversion to the acylglucuronide obtained with 60 mM UDPGA).

The conversion to the acylglucuronide (**3**) did not increase significantly at temperatures above 25 °C, whereas the *O*-glucuronide was generated with more efficiency with rising temperatures (Figure 4). The temperature 25 °C was chosen for the preparative

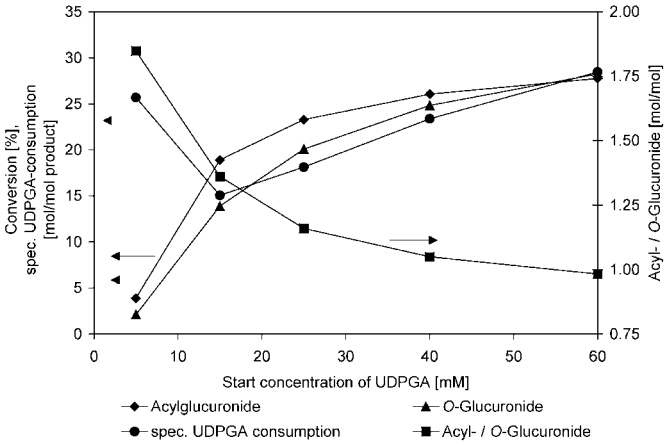

Figure 3. Glucuronidation of mycophenolic acid **(1)** as a function of the UDPGA concentration (pH 7, 37 °C, 20% liver homogenate).

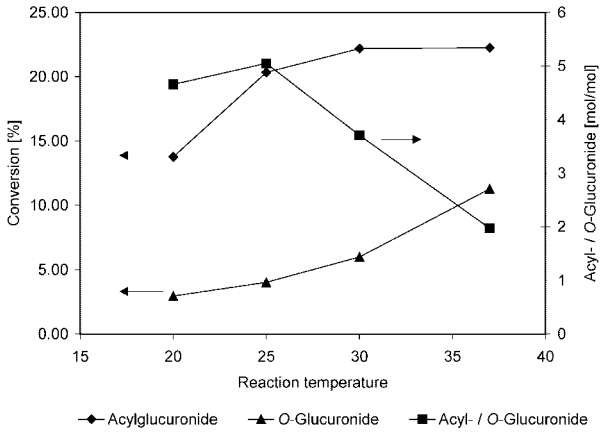

Figure 4. Glucuronidation of mycophenolic acid (**1**) under variation of the reaction temperature (pH 7, 40 mM UDPGA, 20% liver homogenate).

reactions because of the maximum acyl-/*O*-glucuronide ratio and because acylglucuronides are more stable at lower temperatures.[13]

O-Glucuronide synthesis under catalysis of crude bovine liver homogenate showed a clear optimum for the biocatalyst concentration.[14] In this respect, we investigated the dependence of the mycophenolic acid glucuronidation on the concentration of horse liver homogenate (see Figure 5). The formation of both the acyl- and the *O*-glucuronide showed an optimum at 20% of liver homogenate, which was chosen for the further experiments. The ratio between acyl- and *O*-glucuronide formed increased with decreasing catalyst concentration, especially in the range below 20%.

The following additives were tested for stabilisation of the biocatalyst: EDTA – 1 and 5 mM, phenylmethanesulfonyl fluoride – 0.1 mM, PEG 4000 – 10 and 20% w/v, PEG 8000 – 5, 10, 15, and 20% w/v, glycerol – 10, 20 and 42.5% w/v, dithiothreitol – 1 and 5 mM. Regardless of the supplement used, no significant stabilisation of the UGT activity was achieved. Lower conversion resulted in the presence in the reaction mixture of EDTA at 1 and 5 mM, and of the polar additives at higher concentrations (PEG 4000 at 20%, PEG 8000 at 10, 15, 20%, glycerol at 20, 42.5%). Immobilisation was not tested, which efficiently enhanced the storage stability of partially purified UDPGA-glucuronosyl transferase from rat.[15]

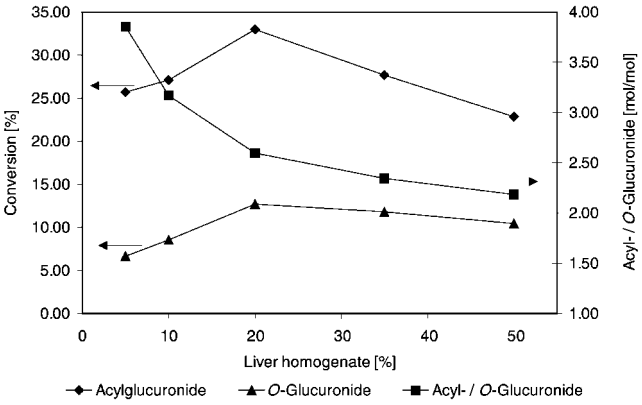

Figure 5. Glucuronidation of mycophenolic acid (**1**) with different concentrations of horse liver homogenate (pH 7, 40 mM UDPGA, 25 °C).

In Table 2 acylglucuronide synthesis is compared before and after reaction optimisation. The optimised conditions were: pH 7.0, UDPGA 25 mM, 25 °C, and 20% horse liver homogenate. Before optimisation, the UDPGA concentration and the reaction temperature had been 40 mM and 37 °C, respectively. On preparative scale with 0.45 g of mycophenolic acid, a 2.8-fold increase in the conversion to the acylglucuronide (**3**) and a 5.8-fold rise of the ratio of acyl- to *O*-glucuronide was achieved.

Table 2. Production of the acylglucuronide (**3**) of mycophenolic acid (**1**) under optimised and non optimised conditions.

Reaction conditions	Analytical scale (100 μL)		Preparative scale (250 – 300 mL)	
	Conversion to acylglucuronide [%]	Acylglucuronide: *O*-glucuronide	Conversion to acylglucuronide [%]	Acylglucuronide: *O*-glucuronide
Initial	34	1.5 : 1	19	0.84 : 1
Optimised	55	3.9 : 1	54	4.9 : 1

A further improvement of acylglucuronide formation might be obtained by optimising the type of co-solvent and its concentration used for solubilising the substrate, which was demonstrated to be an important parameter, e.g., for the glucuronidation of a cholesterol absorption inhibitor.[16]

We also performed the reaction continuously under partially optimised conditions in a 10-mL enzyme membrane reactor. This technology had already been used successfully for the preparation of phenolic glucuronides.[17] Hereby, the substrate solution was continuously pumped through the reactor, and the non-immobilised enzyme from the liver homogenate was retained by an ultrafiltration membrane. In the first experiment steady-state conditions could be held constant during 4 days resulting in a conversion to the acylglucuronide of 18% and a 4:1 ratio of acyl- to *O*-glucuronide. The specific catalyst consumption (mL of liver homogenate per mol of acylglucuronide produced) was reduced 8 times by reusing the enzyme trapped in the reactor. However, since the degree of conversion was considerably lower than in the batch experiments, raw material costs were higher because of the elevated specific UDPGA consumption.

Conclusion

These studies demonstrated that by using crude horse liver homogenate as a cheap and easily accessible biocatalyst, the acylglucuronide of mycophenolic acid could be synthesised with 51% conversion on a preparative scale. Screening for a suitable biocatalyst, however, was inevitable to obtain an efficient process.

Experimental Section

Materials

Chemicals were generally supplied by Fluka Co., Sigma Co. (both Buchs, Switzerland) and Merck Co. (Darmstadt, Germany). UDPGA was purchased from Yamasa Co. (Tokio, Japan), Mycophenolic acid sodium salt and XAD16 were from Fluka. Fresh liver was obtained from local slaughter houses except for rat and dog liver, which were derived from laboratory animals sacrificed in animal trials within Novartis Pharma. The enzyme membrane reactor was constructed according to the specifications of the Research Centre Jülich, Institute for Biotechnology 2 (Jülich, Germany), and was equipped with an ultrafiltration membrane type YM5 with cut-off 5000 Dalton [Amicon Inc., Boston (Mass.) USA].

Preparation of Liver Homogenates

Liver homogenates were prepared by cutting liver into small pieces, mixing 1 g of liver with 1 mL of ice-cold 0.9% NaCl solution, and homogenising in a Braun Potter S tissue homogeniser (B. Braun Biotech Co., Melsungen, Germany) with a 30-mL vessel cooled in ice/water. The homogenate was centrifuged in a Superspeed centrifuge (Du Pont Co, Wilmington, Delaware, USA) using a SS34 rotor at 4 °C for 5 min at 6,000 rpm and subsequently for 10 min at 10,000 rpm. The supernatant was used as biocatalyst and stored at − 80 °C.

Enzymatic Glucuronidation/HPLC-DAD

Analytical assays for enzymatic glucuronidation under the initial conditions before optimisation were set up as follows: HEPES-NaOH buffer pH 7 1 M 13 μL, $MgCl_2$ 0.35 M 10 μL, UDPGA 0.2 M in 20 mM HEPES, pH adjusted to 7, 20 μL, filled up with water to 75 μL, mycophenolic acid sodium salt 0.1 M in DMSO 5 μL, and liver homogenate 20 μL, total volume 100 μL.

The assays were prepared in Eppendorf caps and incubated for 20 h at 37 °C and 800 rpm in an Eppendorf "Thermomixer compact" (Eppendorf Co., Hamburg, Germany). The reactions were stopped by addition of 100 μL of acetonitrile, rigorous mixing, and incubation at room temperature for 5 – 10 min. After centrifugation for 4 min in a Heraeus Biofuge at 14,000 rpm the supernatant was diluted 6-fold with 50% acetonitrile in 0.1% v/v formic acid and subjected to HPLC-MS analytics.

HPLC-DAD analytics were performed on an Agilent 1100 chromatographic system (Agilent Technologies, Waldbronn, Germany) under the following conditions: Column Chromolith Performance RP-18e 100 – 4.6 mm, solvent A = 3 mM H_3PO_4, B = acetonitrile, gradient 5 – 100% B in 4.75 min with flow rate 4 mL/min, detection at 200 – 400 nm.

The conversion (%) was calculated as follows: HPLC peak area of glucuronide/peak area of mycophenolic at start (concentration 5 mM) × 100, peak areas measured at 220 nm.

Stability Studies

Analytical conversion assays under initial reaction conditions (see above) but without UDPGA were preincubated at 37 °C for 44 h (EDTA, PMSF) or at 60 °C for 30 min (other additives). Then the test mixtures incubated at 60 °C were cooled on ice. Glucuronidation at 37 °C was started by UDPGA addition.

Preparative Synthesis and Purification of Acyl- (3) and *O*-Glucuronide (2)/NMR Spectroscopy/HPLC-MS

For preparative synthesis the analytic reactions described above were scaled up linearly to 450 mg of Na^+ mycophenolate (**1**) and performed in nine 50-mL polypropylene vessels under gentle magnetic stirring. After overnight incubation, the reaction mixture was acidified to pH 2.5 with formic acid, and then 1.67% w/v of adsorber resin XAD16 was added. After further incubation under gentle shaking in a laboratory shaker for 30 min the resin was filtered off and extracted 3 times with each 300 mL acetonitrile. The solvent was removed under reduced pressure at 30 °C and the residue subjected to preparative HPLC under the following conditions: Column Chromasil C18 7 μm 100 Å 50 × 500 mm from Eka Chemicals

AB (Bohus, Sweden), solvent A = 0.1% HCOOH, B = acetonitrile, gradient 5 – 80% B in 50 min with flow rate 80 mL/min, detection at 215 nm. 240 mg of acylglucuronide (**3**) (purity > 95%, 34% yield) and 14 mg of 97% pure *O*-glucuronide (**2**) were isolated. ^{1}H NMR (CD_3CN, 400 MHz) of *O*-glucuronide (**2**): δ = 1.80 (br s, 3H,) 2.22 (s, 3H), 2.26 (br t, 2H), 2.38 (t, 2H), 3.46 (t, 1H), 3.50 (t, 1H), 3.56 (t, 1H), 3.62/3.42 [AB(X), 2H], 3.76 (d, 1H), 3.78 (s, 3H), 5.23 (d, 1H), 5.24 (m, 1H), 5.26 (AB, J = 15.5 Hz, 2H); ^{1}H NMR (CD_3CN, 400 MHz) of acylglucuronide (**3**): δ = 1.82 (br s, 3H,) 2.15 (s, 3H), 2.31 (br t, 2H), 2.50 (t, 2H), 3.38 (br d, 2H), 3.44 (t, 1H), 3.33 (t, 1H), 3.53 (t, 1H), 3.38 (br d, 2H), 3.91 (d, 1H), 5.47 (d, 1H), 5.25 (s, 2H), 5.26 (m, 1H), 7.7 (b); HPLC-MS of *O*- (**2**) and acylglucuronide (**3**): Phoenix 40 pump (ThermoFinnigan, San Jose, CA, USA), X-Terra C18 capillary column 150 × 0.4 mm, 3.5 μm (self made), mobile phase A = water-acetonitrile (95/5), B = acetonitrile-water (95/5), both with 1 mM ammonium acetate, elution with 10% B for 2 minutes, then gradient to 90% B in 30 minutes, flow rate 6 μL/min, mass spectrometer Finnigan LCQ-Deca ion trap (ThermoFinnigan) in ESI negative ion mode, spray voltage 4.5 kV, ion transfer tube temperature 300 °C, sheath gas flow rate 45 (arbitrary units), relative collision energy for collision-induced dissociation (CID) 30%, isolation width 2 amu. R_t *O*-glucuronide (**2**) = 12.6 min, r_t acylglucuronide (**3**) = 11.8 min. The MS^2 CID mass spectrum of the *O*-glucuronide (**2**) ($M - H^- = m/z$ 495) showed one dominant product ion at m/z = 319 ($M - H^- - 176$, base peak). This fragment ion was also observed in abundance in the CID spectrum of the acylglucuronide (**3**). Additionally, for this compound (**3**) a dominant product ion at m/z = 305 (base peak) was observed.

Enzyme Membrane Reactor Experiments

The stainless steel reactor had a reaction volume of 10 ml and was stirred magnetically at 300 rpm. The substrate solution was delivered through 2 sterile filters (0.2 μm pore diameter) by a P-500 pump (Amersham Biosciences, Freiburg, Germany) at 1.2 mL/min flow rate (=500 min residence time). Sterilisation had been executed before by flushing the system with 0.1% peracetic acid overnight at a flow rate of 0.2 mL/min. Partially optimised reaction conditions were applied: pH 7, 40 mM UDPGA, 20% horse liver homogenate, 25 °C.

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