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Glucuronide production by whole-cell biotransformation using genetically engineered fission yeast *S. pombe*

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Running title page

Glucuronide production by UGT expressing fission yeast

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Nonstandard abbreviations: 4MU, 4-methylumbelliferone; 4MUG, 4-methylumbelliferone-ß-D-glucuronide; T, testosterone; TG, testosterone glucuronide; UDP-GA, UDP-glucuronic acid; UGDH, UDP glucose-6-dehydrogenase; UGT, UDP glycosyltransferase.

Abstract

Drug metabolites generated by UDP glycosyltransferases (UGTs) are needed for drug development and toxicity studies, especially in the context of safety testing of metabolites during drug development. Since chemical metabolite synthesis can be arduous, various biological approaches have been developed; however, no whole-cell biotransformation with recombinant microbes that express human UGTs was yet achieved. In this study we expressed human UDP glucose-6-dehydrogenase (UGDH) together with several human or rat UGT isoforms in the fission yeast *Schizosaccharomyces pombe* and generated strains that catalyze the whole-cell glucuronidation of standard substrates. Moreover, we established two methods to obtain stable isotope-labeled glucuronide metabolites: The first uses a labeled aglycon, while the second employs $^{13}C_6$-glucose as a metabolic precursor of isotope-labeled UDP-glucuronic acid (UDP-GA) and yields a sixfold labeled glucuronide. The system described here should lead to a significant facilitation in the production of both labeled and unlabeled drug glucuronides for industry and academia.

Introduction

The metabolic steps that lead to drug clearance in the human body are divided into two distinct parts, which encompass chemical modifications of the parent compound (phase I) and conjugations of parent or phase I metabolites with endogenous molecules (phase II). As a huge majority of the 200 most prescribed drugs in the United States are metabolized in the human body (Williams et al., 2004), the synthetic or biosynthetic accessibility of drug metabolites is a prerequisite for drug development and toxicity studies. According to current knowledge, cytochrome P450 (CYP) systems are most important for phase I (Bernhardt, 2005; Ingelman-Sundberg et al., 2007) and UGTs for phase II reactions (Mackenzie et al., 2005), respectively. The human UGT superfamily consists of four families with 22 isoenzymes: The 19 members of the UGT1 and UGT2 families (the latter encompassing subfamilies 2A and 2B) are primarily involved in xenobiotic metabolism and efficiently utilize uridine diphosphate glucuronic acid (UDP-GA) for the conjugation of drugs to glucuronic acid (Mackenzie et al., 2005). UGT3A1 was recently shown to be a UDP-N-acetylglucosaminyltransferase that also appears to have a function in drug metabolism (Mackenzie et al., 2008), the catalytic activity of UGT3A2 is not yet known, and UGT8A1 catalyzes the transfer of galactose from UDP-galactose to ceramide (Bosio et al., 1996). Similar to many CYPs some drug metabolizing UGT isoforms (e.g. UGT1A1, UGT1A6, UGT1A9 and UGT2B15) display polymorphisms with a demonstrated association between genotype and clinical pharmacokinetics (Katz et al., 2008). Many UGTs are expressed in the liver, but other drug entry points like epithelial surfaces of the nasal mucosa, gut, skin, brain, prostate, uterus, breast, placenta and kidney also host UGT activity (Tukey and Strassburg, 2000). UGT enzymes are integrally associated with the membranes of the endoplasmic

reticulum, possibly with most part of the protein being oriented towards the luminal side, and may form homo- and heterooligomeric structures (Bock and Köhle, 2009).

The formation of glucuronide metabolites may pose toxicological problems to patients via increased drug activity upon glucuronidation (Coller et al., 2009) or cause dosage complications due to the uncontrolled re-release of parent aglycones by systemic or enteric β-glucuronidase activity (Prueksaritanont et al., 2006). In addition, acyl-glucuronides can exhibit chemical reactivity and can readily form covalently bound adducts to protein. Such protein adducts have been discussed as a cause for idiosyncratic adverse drug reactions (Bailey and Dickinson, 2003). The identification and structure elucidation of drug glucuronides can be accomplished with milligram amounts, but safety testing may demand gram quantities. As classical chemical synthesis of glucuronide metabolites can be cumbersome, various biological techniques have been developed to this aim, which include metabolite isolation from animal body fluids, the use of liver homogenates or liver microscale culture, and enzyme preparations obtained after recombinant expression of metabolizing enzymes in cell culture or microbial systems (Radominska-Pandya et al., 2005; Khetani and Bhatia, 2008). However, each of these methods has its specific drawbacks. For instance, human liver microsomes contain native UGTs, but their use may be hampered by their relative scarce availability, batch-to-batch variations and the absence of UGTs that are only expressed in other tissues (Tukey and Strassburg, 2000; Jia and Liu, 2007). Heterologous expression of human UGTs in various mammalian or insect cell lines was very helpful for the determination of basic kinetic parameters of the enzymes, but suffers from low expression levels, low activity normalized to biomass and stability problems (Radominska-Pandya et al., 2005; Trubetskoy et al., 2008). And while functional UGT expression in bakers yeast has been shown *per se*, glucuronide production could only be achieved after preparation of yeast microsomes and addition of UDP-GA. The use of the costly cofactor UDP-GA at

considerable concentrations in existing preparative methods seems to be necessary due to the endoplasmic membrane barrier which limits the entry of UDP-GA in the lumen. In addition, the use of microsomal preparations implies the presence of many UGT isoforms and of other systems that may reduce the yield of the desired glucuronide product by cofactor competition.

Whole-cell biotransformations with recombinant microbes offer many advantages with respect to scalable metabolite production, and corresponding expression systems for human CYPs have thus been established in bacteria and yeasts (Ghisalba and Kittelmann, 2007; Pscheidt and Glieder, 2008). In recent years, we demonstrated the usefulness of recombinant strains of the fission yeast *Schizosaccharomyces pombe* that express human CYPs for the production of CYP metabolites of illicit drugs (Peters et al., 2009) and doping substances (Zöllner et al., in press). Since no glucuronidation by whole-cell biotransformation with an unicellular organism that recombinantly expresses human UGTs was yet reported, it was the aim of this study to develop such a system using *S. pombe*.

Materials and Methods

Fine chemicals. 4-Methylumbelliferone (4MU), testosterone (T) and testosterone glucuronide (TG) potassium salt were purchased from Sigma-Aldrich (Hamburg, Germany), 4-methylumbelliferone-ß-D-glucuronide (4MUG) dihydrate was from Carl Roth (Karlsruhe, Germany), $^{13}C_6$-glucose was from euriso-top (Gif-Sur-Yvette, France). Deuterated T was synthesized by toroma organics Ltd. (Saarbrücken, Germany). All other chemicals used were either from Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich (Hamburg, Germany). Methanol (HPLC-grade) was from Fisher scientific (Loughborough, UK).

Coding DNA sequences. The cDNAs of human UGT1A1, UGT1A9, UGT2A1, UGDH and or rat UGT1A7 (rUGT1A7) were synthesized by Entelechon GmbH (Regensburg, Germany); cDNAs of human UGT1A6, UGT1A7, UGT1A8, UGT1A10, UGT2B15 and UGT2B17 were synthesized by GeneArt GmbH (Regensburg, Germany).

Media and general techniques. We used general DNA manipulating methods as well as media and genetic methods for fission yeast as described before (Drăgan et al., 2005). In addition, we used EMM containing 100 g L^{-1} glucose for biotransformation assays and EMM containing 20 g L^{-1} $^{13}C_6$-glucose for the synthesis of isotope-labeled glucuronides.

Construction of fission yeast strains. UGT cDNAs were cloned via *Nde* I and *Bam*H I into the integrative vector pCAD1 (Drăgan et al., 2005) that integrates into the *leu1* gene of the fission yeast genome, thereby compensating an *ura4* defect. UGDH cDNA was cloned into the expression vector pREP1 (Maundrell, 1993) using *Nde* I and *Bam*H I yielding pREP1-UGDH. The correctness of all constructs was verified by automatic sequencing (MWG-Biotech; Ebersberg, Germany). The construction of fission yeast strains expressing functional UGTs was done in two steps. pCAD1-UGT-constructs were prepared, prior to transformation, as reported previously (Drăgan et al., 2005), and used to transform yeast strain NCYC 2036

(*h⁻ura4-D18*). Transformation was done, using competent cells prepared as described elsewhere (Suga and Hatakeyama, 2005) and yielded strains CAD200, DB1, DB3, DB5, DB23, DB24, DB25, DB26, DB32 and DB33. Correct integration into the *leu1* locus was verified by selection of leucine auxotrophs on EMM dishes containing 5 µM thiamine but no leucine. Subsequently, strains containing an integrated UGT expression cassette were transformed with pREP1-UGDH as described (Okazaki et al., 1990) to yield strains CAD203, DB11, DB13, DB15, DB43, DB44, DB45, DB46, DB52 and DB53. Both fission yeast expression vectors used in this study contain the strong endogenous *nmt1* promotor (Maundrell, 1990; Maundrell, 1993) that permits expression regulation via the presence or absence of thiamine in the media. Therefore, transformed cells were selected by plating on EMM dishes with 5 µM thiamine to allow better growth under repressed conditions. All yeast strains used in this study are available from PomBioTech GmbH.

Biomass production. All cultures were set up in absence of thiamine in order to induce expression by the *nmt1* promoter. Incubation was carried out at 30°C and 150 rpm. 10 mL EMM containing the appropriate supplements and lacking thiamine were inoculated with cells grown on a dish for three days and incubated to stationary phase; these cells were then used to inoculate 100 mL main cultures. Main cultures were incubated for one to two days for the parental strains NCYC2036, CAD200, DB1, DB3, DB5, DB23, DB24, DB25, DB26, DB32 and DB33; and three to five days for the coexpressing strains CAD203, DB11, DB13, DB15, DB43, DB44, DB45, DB46, DB52 and DB53.

Whole-cell biotransformation assay. The biomass was centrifuged (3.000 *g*, 5 min, RT) and resuspended in 12 mL EMM with 100 g L^{-1} glucose and supplements as required. Substrate was added to a final concentration of 500 µM by adding 600 µL of 10 mM stock solutions in ethanol. The biotransformations were carried out in 250 mL wide-neck erlenmeyer flasks for 72 h at 30°C and 150 rpm. Sample volumes of 2 mL were taken at 0 h and 72 h and

centrifuged (10.000 *g*, 5 min, RT). The cell-pellets were used to determine the biomass dry weight while the supernatants were centrifuged again and then used for HPLC and LC/MS analysis. All results shown were obtained in at least three independent experiments.

Synthesis of isotope-labeled glucuronide metabolites. Due to the high cost of the labeled compounds these experiments were done at 1 mL scale. For the comparison of the biosynthesis of labeled 4MUG to that of unlabeled 4MUG, the cultivation of the cells was done in EMM containing either $^{13}C_6$-glucose or unlabeled glucose at a concentrations of 20 g L^{-1}. 5 mL of media without thiamine were inoculated with cells of strain DB13 and incubated for one day at 30 °C and 150 rpm. 1 mL of this culture was then used to set up a 10 mL main culture which in turn was incubated for three days under the same conditions. The biomass was harvested by centrifugation (3.000 *g*, 5 min, RT) and cells were resuspended in 1 mL of EMM containing a final concentration of 500 µM of 4MU (50 µL of an ethanolic 10 mM 4MU stock solution). The assay was carried out in a 96-deep-well plate for 24 h at 30 °C and 750 rpm in triplicates. For the synthesis of stable isotope-labeled TG the biotransformation assay was carried out with strain DB53 as described before using T doubly deuterated at C-2, deuterated in β and α positions at C-4 and C-6, resp., and deuterated at the C-17's hydroxyl group as substrate. In the acidic fission yeast media, rapid exchange of the D atom in the OD-group at C-17 leads to the fourfold labeled substrate as shown in Figure 2a. All samples were prepared as described above and analyzed by HPLC and LC/MS.

HPLC-analysis. HPLC was performed using a Series II 1090 system (Hewlett Packard, USA) equipped with a Lichrospher 100 column (125 × 4,6 mm, RP18, 5 µM; Merck, Darmstadt, Germany) and a diode array detector. The flow rate was 1 mL min^{-1} and the column temperature was 40 °C. For the simultaneous detection of 4MU and 4MUG the initial mobile phase composition was 85 % 0.1 % acetic acid (A) and 15% methanol (B). B was linearly raised to 55 % from 5 to 10 min, maintained at 55 % for further 2 min and then

immediately returned to 15 % until the end of the run at 15 min. The eluents were monitored at 320 nm. The retention times of 4MU and 4MUG were 12.4 min and 8.4 min, respectively. Quantification of 4MUG was done using an external 4MUG standard, prepared in the initial mobile phase at a concentration of 200 µM.

For the simultaneous detection of T and TG, the initial mobile phase composition was 85 % 0.1 % acetic acid (A) and 15 % methanol (B). B was linearly raised to 90 % from 2 min to 12 min and then immediately returned to 15 % B until the end of the run at 17min. The eluents were monitored at 240 nm. The retention times of T and TG were 13.3 min and 11.5 min, respectively. TG concentrations were quantified using an external T standard at a concentration of 200 µM.

Liquid chromatography-mass spectrometry. Samples were analyzed by nanospray ionization high resolution mass spectrometry (FTMS) in the positive ion mode using a linear ion trap/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Germany) equipped with a Triversa Nanomate^{TM} nanospray ion source (Advion, USA). The instrument was coupled to the post-column flow of an Agilent 1100 HPLC detection system (Agilent, Germany) equipped with a YMC ODS-AQ column (150 × 4 mm. 5 µm, YMC, YMC Germany). The flow rate was 800 µL min^{-1} and the column temperature was 40 °C. A linear gradient from 10% to 90% acetonitrile vs. water containing 50 mM formic acid was applied from 0 to 14 min, maintained at 90% for further 3 min and then immediately returned to the initial conditions for 3 min.

The bulk of the LC flow (700 µL min^{-1}) was discarded and approximately 100 µL min^{-1} was passed to the nanospray source. The source voltage and capillary voltage were set to 1.5 kV and 22 V, respectively. Nitrogen was used as bath gas to slow down the motion of the ion population in the C-trap. Collision-induced dissociation experiments (FTMS^{n}) were performed in the linear ion trap with collision energies of 26% normalized collision energy at

activation Q values of 0.250 with helium as collision gas. The isolation width for CID experiment in the linear ion trap was set to ± 1 amu. The FTMS mass analyzer was operated at a resolution of approximately 60,000 FWHM at m/z 400 in the full scan MS mode and approximately 15,000 FWHM at m/z 400 in the full scan MS^n mode. High-resolution mass spectra were acquired in the range of m/z 50 to 800. Exact mass measurements in the FTMS- and FT-MS^n mode were performed after external calibration using the manufacturer's calibration mixture prior LC-$FTMS^n$ investigations.

Results

Synthetic cDNA sequences coding for nine wildtype human UGTs or rat UGT1A7, repsectively, were each cloned into the integrative fission yeast expression vector pCAD1 (Drăgan et al., 2005). Transformation of fission yeast strain NCYC2036 with the resulting constructs yielded the first set of strains, each of which expresses a single mammalian UGT isoform (see Table 1). The newly created strains grew normally and did not display a visible phenotype (data not shown). As mentioned above, UGT1 and UGT2 isoenzymes require UDP-GA as a cofactor which is synthesized from UDP glucose by UGDH in mammals. While the formation of UDP glucose in yeasts is known, wild-type bakers yeast does not produce UDP-GA (Oka and Jigami, 2006) and the fission yeast genome contains no open reading frame that shows significant homology to mammalian UGDH enzymes (Wood et al., 2002). Still, the presence of an endogenous UGDH activity due to a non-homologous fission yeast protein could not *a priori* be ruled out. But incubation of human UGT expressing strains (Table 1) with either 4MU (Uchaipichat et al., 2004) or T (Sten et al., 2009) as standard substrates did not lead to any detectable glucuronide formation (data not shown). For the development of a whole-cell biotransformation process an endogenous source of UDP-GA therefore had to be provided by coexpression of UGDH (see Figure 1). A synthetic cDNA coding for human UGDH was cloned into the autosomally replicating expression vector pREP1 (Maundrell, 1993) to yield the new plasmid pREP1-hUGDH. Transformation of all UGT expressing strains with this plasmid yielded a new set of ten UGT and UGDH coexpressing strains (Table 1). These new strains showed significantly reduced growth under induced expression conditions and yielded biomass concentrations between 1 g L^{-1} and 2 g L^{-1} after three to five days in contrast to biomass yields between 2 g L^{-1} and 3 g L^{-1} for strains that only express UGTs (data not shown). Whole-cell biotransformation assays demonstrated that

all coexpressing strains were able to catalyze the glucuronidation of either 4MU or T (Table 2). The identity of the products 4MUG and TG was in each case confirmed by LC/MS analysis (data not shown). As could be expected, the biotransformation activities of the different UGT expressing strains towards 4MU varied from around 1 µmol L^{-1} day^{-1} for UGT1A1 and UGT1A6, resp., up to 150 µmol L^{-1} day^{-1} in the case of UGT1A9. In terms of specific production rates (i.e. normalized to the biomass dry weight), the highest values of almost 20 µmol day^{-1} g^{-1} were achieved by the UGT1A9 and UGT2A1 expressing strains, resp., while the strains expressing UGT1A1, UGT1A6, and rat UGT1A7, resp., yielded only values around or even below 0.1 µmol day^{-1} g^{-1}. Strain DB53 expressing human UGT2B17 produced TG with a maximum space-time yield of 32.6 µmol L^{-1} day^{-1} and a maximum specific production rate of 3.1 µmol day^{-1} g^{-1}. To the best of our knowledge, these data demonstrate for the first time a successful whole-cell biotransformation using recombinant human UGTs expressed in a unicellular organism.

The availability of stable-isotope labeled glucuronides is a prerequisite for the sensitive quantification of glucuronide metabolites in biological matrices by LC-MS/MS, e.g. for the toxicokinetic monitoring of glucuronides during nonclinical safety studies. Having established a functional *in vivo* system for the production of glucuronides, we therefore investigated the possibility to produce isotope-labeled glucuronide metabolites by applying two different strategies. First, either non-labeled T or fourfold deuterated T (D_4-T) were subjected to whole-cell biotransformation with strain DB53 that coexpresses UGT2B17 and UGDH (Figure 2a). The comparison of the LC/MS analysis of the extracted ion currents (EICs) of non-labeled and fourfold labeled TG as well as the respective fragmentation spectra unambigously demonstrate the sucessful formation of D_4-TG (Figure 2b-e). Second, we intended to demonstrate a more general labeling technique by using ^{13}C isotope-labeled glucuronic acid as substrate and strain DB13 (expressing UGT2A1 and UGDH). Based on the

biosynthesis scheme outlined above (Figure 1) which leads to the endogenous formation of UDP-GA in UGDH expressing fission strains, we used $^{13}C_6$- glucose as metabolic precursor of six-fold labeled UDP-GA (Figure 2f). For this purpose, $^{13}C_6$-glucose was added throughout all culturing periods prior to performing the biotransformation in order to deplete ^{12}C-compounds in the central carbon metabolism of the cells as much as possible. Again, the comparison of the LC/MS analysis of the EICs of 4MUG and 4MU-$^{13}C_6$G as well as the respective prove the sucessful formation of the sixfold labeled product (Figure 2g,i). In this case the main signal of the fragmentation spectra is not changed as it corresponds to the unlabelled aglycon (Figure 2h, j). These results show that the generation of isotope-labeled glucuronide metabolites can be conveniently achieved *in vivo* using the fission yeast system presented in this study, and that according to requirements either the aglycon or the sugar moiety may be labeled.

Discussion

In this study, ten fission yeast strains were cloned that express either one of nine human UGTs or rat UGT1A7, respectively (Table 1). As fission yeast does not have an endogenous UGDH enzyme (Wood et al., 2002), neither of these strains displayed glucuronidation activity towards 4MU or T due to a lack of UDP-GA (data not shown). Therefore, a second set of strains was created that coexpress the UGTs mentioned above together with human UGDH. In addition to the intracellular production of UDP-GA as such, its subcellular localization had also to be considered: In human cells, UDP-GA is formed by UGDH in the cytoplasm and subsequently transported by nucleotide sugar transporters into the lumen of the endoplasmic reticulum, where the UGTs are located (Kobayashi et al., 2006). If after expression in fission yeast the subcellular localization of both UGTs and UGDH is corresponding to their targeting in mammalian cells, then for biotransformation to occur at least one of the endogenous nucleotide sugar transporters (like *vrg4* or *hut1* (Nakanishi et al., 2001)) must be able to transport UDP-GA into the ER lumen. This seems indeed to be the case, as all coexpressing strains were able to catalyze the glucuronidation of either 4MU or T (Table 2). The time space yield of the different UGT expressing strains towards 4MU varied by roughly two orders of magnitude from around 1 µmol L^{-1} day^{-1} up to 150 µmol L^{-1} day^{-1}. With respect to the relative activity of some of the UGT isoforms (e.g. UGT1A6 vs. UGT1A9), these results are partially at variation to earlier results obtained with enzymes purified from transfected human embryonic kidney cells (Uchaipichat et al., 2004). However, the very different experimental settings are likely to account for some of these variations. Strain DB53 expressing human UGT2B17 produced TG with a maximum space-time yield of 32.6 µmol L^{-1} day^{-1} and a maximum specific production rate of 3.1 µmol day^{-1} g^{-1}. Thus, a successful whole-cell biotransformation using recombinant human UGTs expressed in a unicellular organism could be established for the first time. In comparison to the alternatives mentioned above, a

significant benefit of this system is its self-sufficiency with respect to the expensive cofactor UDP-GA. A further advantage is its cost-effective scalability due to the endogenous generation of all reaction constituents except for the substrate. While fission yeast is at present not a widely used organism in biotechnology, its widely tolerable pH range makes adaptation to substrate and product requirements easily achievable; for instance, the production of (notoriously unstable) acyl glucuronides may be done under acidic conditions to avoid rapid degradation (Ebner et al., 1999). And in addition to up-scaling, scaling down could lead to a simple and efficient high-throughput screening method for large scale UGT profiling or inhibition studies using living cells.

As the availability of stable-isotope labeled glucuronides is desirable for the LC-MS/MS analysis of glucuronide metabolites in biological matrices, we established two different methods for their preparation. In the first appraoch a labeled aglycon served as a substrate, as in a recent study where pooled human liver microsomes were used for a similar biotransformation (Turfus et al., 2009). In this case, fourfold deuterated T was sucessfully glucuronidated by strain DB53 (Figure 2a-e). While this approach for the production of stable-isotope labeled glucuronides is very straightforward, it depends on the availability of a sufficiently labeled aglycon, which may not always be at hand. Moreover, in some instances unfavorable isotope effects may occur. Therefore, a second strategy was developed that employs $^{13}C_6$-glucose as a metabolic precursor, which is converted within the UGDH expressing fission yeast strains to $^{13}C_6$-labeled UDP-glucuronic acid (Figure 1). Exemplarily, strain DB13 (expressing UGT2A1 and UGDH) was sucessfully used for the production of sixfold labeled 4MUG (4MU-$^{13}C_6$G) by this method (Figure 2f-j). In conclusion, in this study we demonstrate the functional expression of human UGDH with nine human and one rat UGT enzymes in fission yeast that can be conveniently used for the synthesis of either labeled or unlabeled glucuronide metabolites by whole-cell biotransformation.

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Footnotes

C.-A.D. and D.Bu. contributed equally to this work.

Figure legends

Figure 1 Schematic representation of the engineered glucuronide biosynthetic pathway in recombinant fission yeast strains. The scheme shows in black the endogenous pathway that leads to the formation of UDP glucose with branching points to several pathways of the central carbon metabolism; reactions depicted in blue indicate the reaction steps introduced into the host by the heterologous coexpression of human UGDH and a mammalian UGT. Glucose (**1**) enters the cell and is converted to glucose-6-phosphate (**2**) by hexokinase (HK), which can be interconverted to glucose-1-phosphate (**3**) by phosphoglucomutase (PGM). The latter is conjugated with UDP by UDP glucose pyrophosphorylase (UGPase) to yield UDP glucose (**4**). Human UGDH oxidizes **4** to UDP-GA (**5**) which then enters the lumen of the endoplasmic reticulum. Eventually, the heterologously expressed mammalian UGT performs the conjugation of **5** to an aglycon (**R**) that passes both plasma and endoplasmic membrane to yield a glucuronide metabolite (**6**). The glucuronide metabolite in turn leaves both the endoplasmic reticulum and the cell.

Figure 2 Production of stable isotope-labeled glucuronides using either a labeled aglycon (**a-e**) or a labeled sugar moiety (**f-j**). Scheme of the glucuronidation of D_4-testosterone (D_4-T) by UGT2B17 (**a**). Comparison of the LC/MS analysis of the extracted ion currents (EIC) of non-labeled (**b**) and fourfold labeled (**d**) testosterone glucuronide and fragmentation spectra of testosterone glucuronide (TG $\rightarrow$ T, $m/z_{theo.}$ = 289.21621, Δ = -0.28 ppm, **c**) and D_4-testosterone glucuronide (D_4-TG $\rightarrow$ D_4-T, $m/z_{theo.}$ = 293.24131, Δ = 0.17 ppm, **e**). Scheme of the glucuronidation of 4-methylumbelliferone (4MU) by UGT2A1 with UDP-$^{13}C_6$-GA (**f**) which is produced from $^{13}C_6$-glucose according to Figure 1. Comparison of the LC/MS analysis of non-labeled (**g**) and sixfold labeled (**i**) 4-methylumbelliferone glucuronide and fragmentation spectra of 4-methylumbelliferone glucuronide (4MUG $\rightarrow$ 4MU, $m/z_{theo.}$ =

177.05462, Δ = -1.35 ppm, **h**) and 4-methylumbelliferone $^{13}C_6$-glucuronide (4MU-$^{13}C_6$G → 4MU, $m/z_{theo.}$ = 177.05462, Δ = -0.57 ppm, **j**).

Figure 3 Fission yeast strain construction scheme. The procedure is exemplarily shown for UGT1A1. The uracil-deficient parental strain NCYC2036 was transformed using the integrative plasmid pCAD1-UGT1A1. The resulting leucin-deficient strain DB1 was in turn transformed with the autosomal plasmid pREP1-UGDH yielding strain DB11. For all other UGT isoforms, the cloning procedure was done accordingly.

Tables

Table 1: Fission yeast strains used in this study.

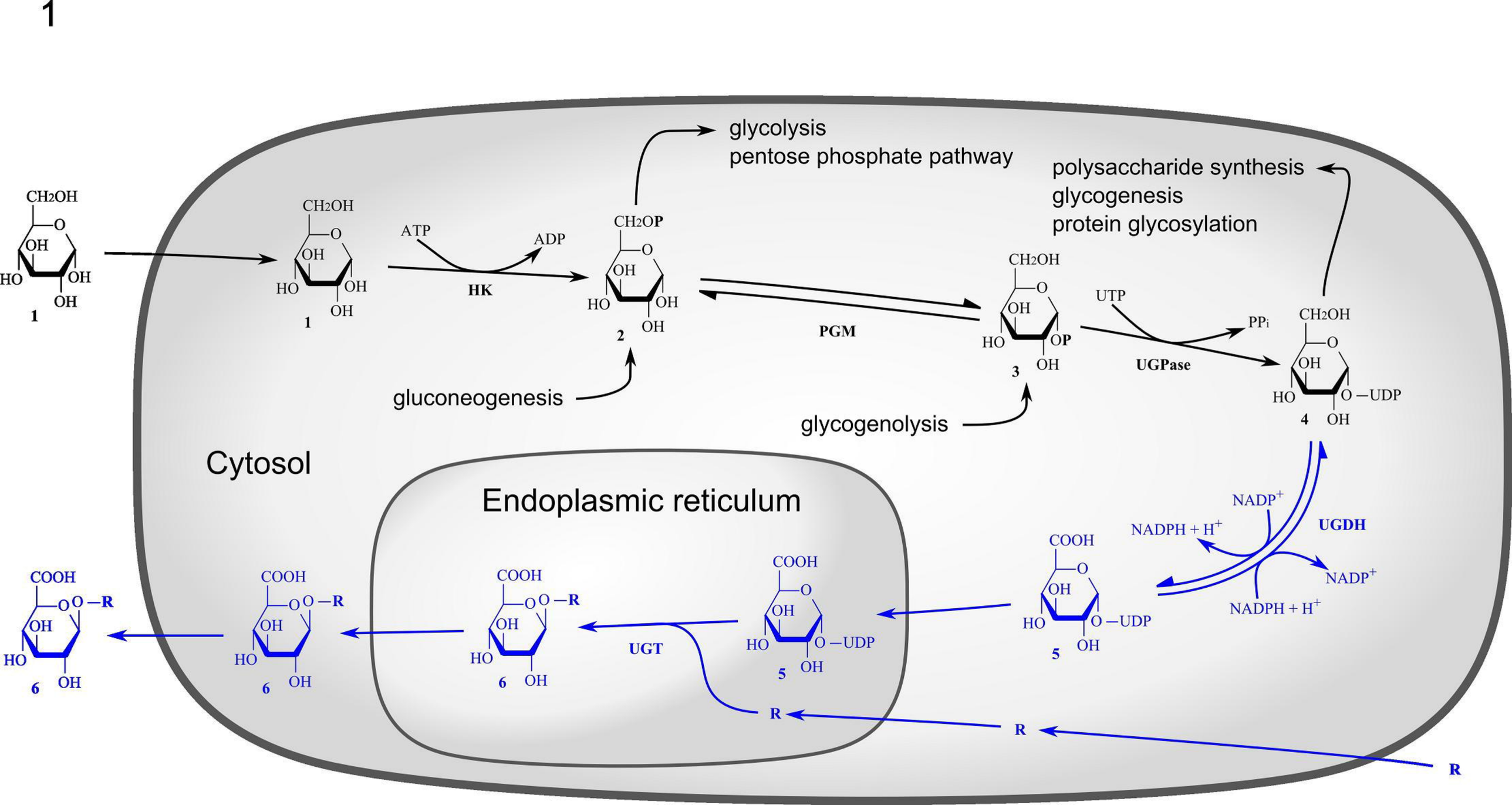
Expressed protein(s)	**Species type**	**Strain name**	**Parent strain**	**Genotype**	**Reference**
		NCYC2036		*h⁻ ura4-D18*	(Losson and Lacroute, 1983)
UGT1A1	human	DB1	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A1*	this study
UGT1A6	human	DB23	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A6*	this study
UGT1A7	human	DB24	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A7*	this study
UGT1A8	human	DB25	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A8*	this study
UGT1A9	human	CAD200	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A9*	this study
UGT1A10	human	DB26	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A10*	this study
UGT2A1	human	DB3	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT2A1*	this study
UGT2B15	human	DB32	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT2B15*	this study
UGT2B17	human	DB33	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT2B17*	this study
rUGT1A7	rat	DB5	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-rUGT1A7*	this study
UGT1A1, UGDH	human	DB11	DB1	*h⁻ ura4-D18 leu1::pCAD1-UGT1A1 / pREP1-UGDH*	this study
UGT1A6, UGDH	human	DB43	DB23	*h⁻ ura4-D18 leu1::pCAD1-UGT1A6 / pREP1-UGDH*	this study
UGT1A7, UGDH	human	DB44	DB24	*h⁻ ura4-D18 leu1::pCAD1-UGT1A7 / pREP1-UGDH*	this study
UGT1A8, UGDH	human	DB45	DB25	*h⁻ ura4-D18 leu1::pCAD1-UGT1A8 / pREP1-UGDH*	this study
UGT1A9, UGDH	human	CAD203	CAD200	*h⁻ ura4-D18 leu1::pCAD1-UGT1A9 / pREP1-UGDH*	this study
UGT1A10, UGDH	human	DB46	DB26	*h⁻ ura4-D18 leu1::pCAD1-UGT1A10 / pREP1-UGDH*	this study
UGT2A1, UGDH	human	DB13	DB3	*h⁻ ura4-D18 leu1::pCAD1-UGT2A1 / pREP1-UGDH*	this study
UGT2B15, UGDH	human	DB52	DB32	*h⁻ ura4-D18 leu1::pCAD1-UGT2B15 / pREP1-UGDH*	this study
UGT2B17, UGDH	human	DB53	DB33	*h⁻ ura4-D18 leu1::pCAD1-UGT2B17 / pREP1-UGDH*	this study
rUGT1A7, UGDH	rat, human	DB15	DB5	*h⁻ ura4-D18 leu1::pCAD1-rUGT1A7 / pREP1-UGDH*	this study

Table 2: Glucuronide metabolite formation rates determined in whole-cell biotransformations of fission yeast strains coexpressing human UGDH and mammalian UGT enzymes.

				Space-time yield (µmol L^{-1} day^{-1})		Specific production rate (µmol day^{-1} g^{-1} biomass dry weight)	
Strain	Expressed protein(s)	Substrate	Product	min	max	min	max
DB11	UGT1A1, UGDH	4MU	4MUG	0.5	1.8	0.04	0.14
DB43	UGT1A6, UGDH	4MU	4MUG	0.6	3.5	0.04	0.22
DB44	UGT1A7, UGDH	4MU	4MUG	8.2	18.2	0.85	2.10
DB45	UGT1A8, UGDH	4MU	4MUG	5.9	23.0	0.43	2.50
CAD203	UGT1A9, UGDH	4MU	4MUG	64.2	151.5	12.10	19.70
DB46	UGT1A10, UGDH	4MU	4MUG	7.0	18.2	0.56	1.50
DB13	UGT2A1, UGDH	4MU	4MUG	22.8	114.5	4.00	19.50
DB52	UGT2B15, UGDH	4MU	4MUG	3.3	7.9	0.24	0.63
DB53	UGT2B17, UGDH	4MU	4MUG	0.0	0.0	0.00	0.00
DB53	UGT2B17, UGDH	T	TG	15.5	32.6	1.20	3.10
DB15	rUGT1A7, UGDH	4MU	4MUG	2.3	2.3	0.16	0.19

4MU: 4-methylumbelliferone; 4MUG: 4-methylumbelliferone glucuronide; T: testosterone; TG; testosterone glucuronide.

1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PGM
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PPi
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glycogenolysis
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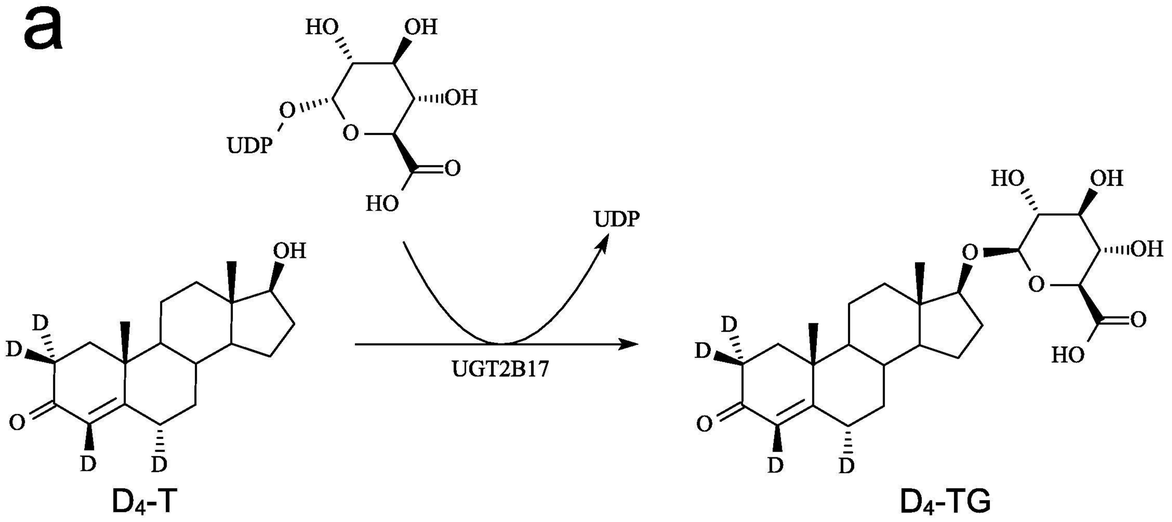
a



D4-T D4-TG

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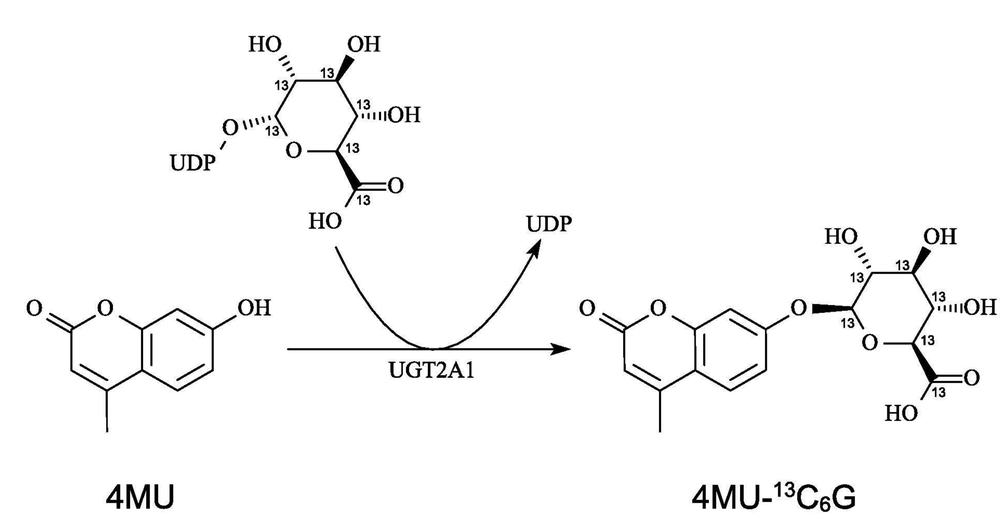

4MU 4MU-$^{13}C_6$G

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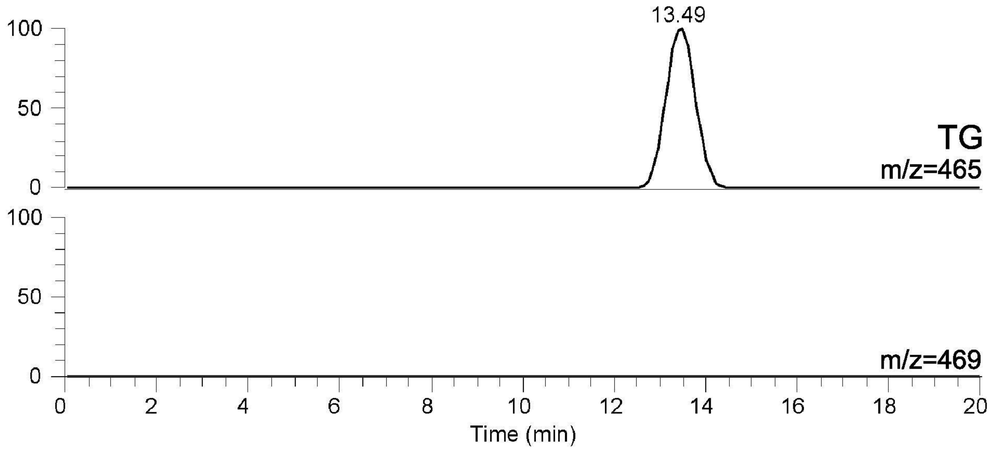

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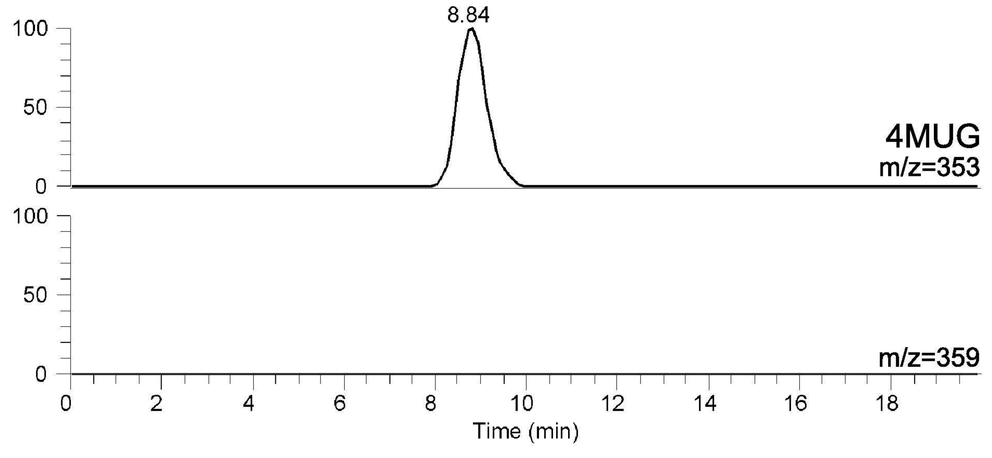

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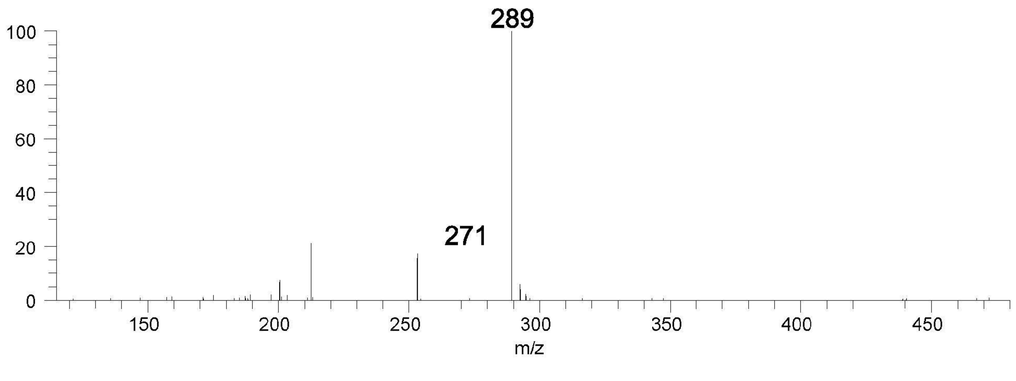

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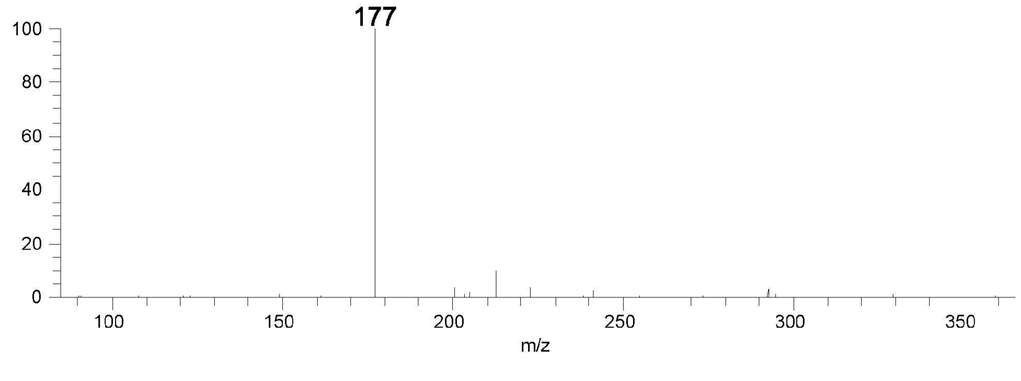

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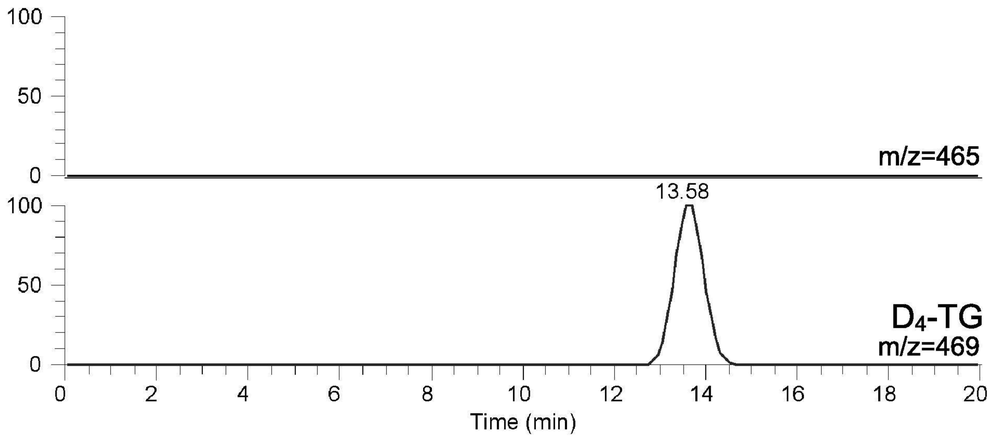

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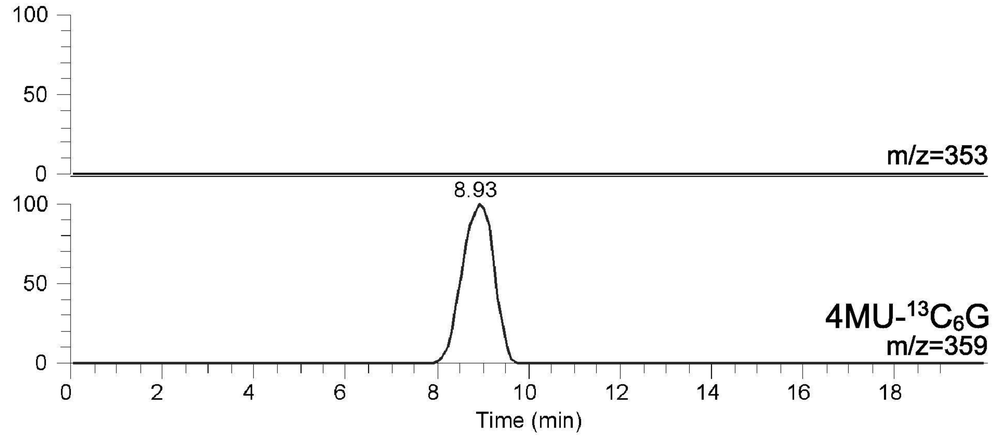

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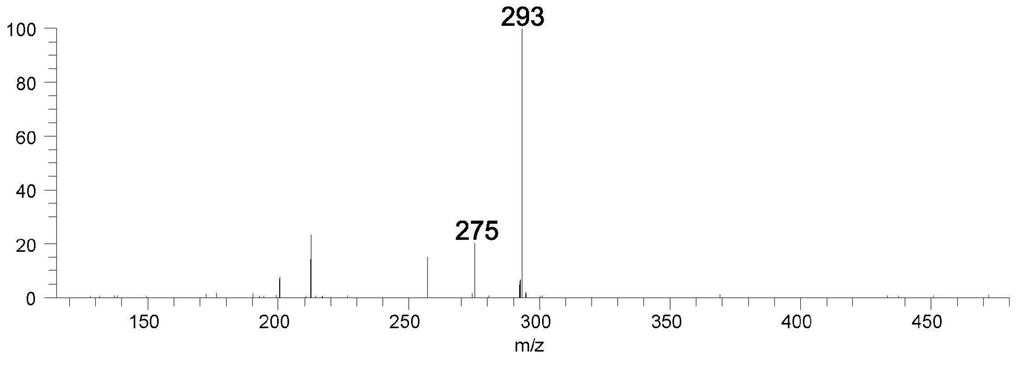

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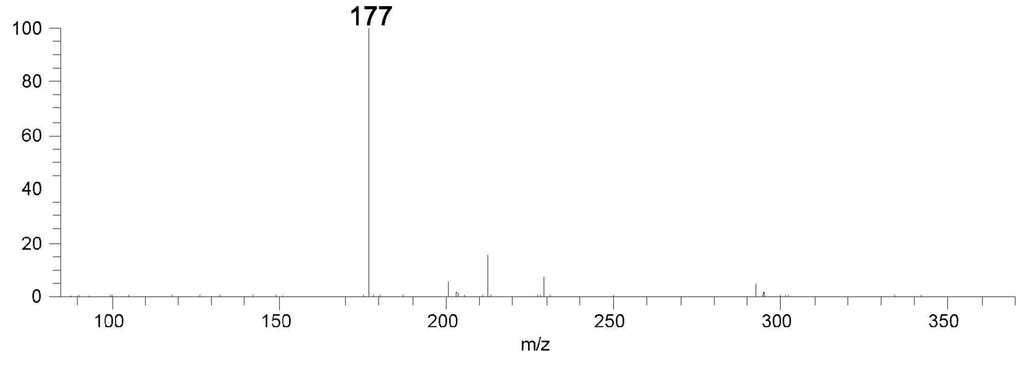

3


NCYC2036
pCAD1-UGT1A1
DB1
pREP1-UGDH
DB11
h- ura4-D18
h- ura4-D18
leu1:: pCAD1-UGT1A1
h- ura4-D18
leu1:: pCAD1-UGT1A1 /
pREP1-UGDH