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Biotechnological synthesis of drug metabolites using human cytochrome P450 2D6 heterologously expressed in fission yeast exemplified for the designer drug metabolite 4′-hydroxymethyl-α-pyrrolidinobutyrophenone☆

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ABSTRACT

The aim of this study was evaluating the principle feasibility of biotechnological synthesis of drug metabolites using heterologously expressed human cytochrome P450 (CYP) enzymes. Human CYP2D6 expressed in fission yeast (*Schizosaccharomyces pombe*) strain CAD58 was used as model enzyme and the designer drug 4′-methyl-α-pyrrolidinobutyrophenone (MPBP) as model drug. For synthesis of 4′-hydroxmethyl-α-pyrrolidinobutyrophenone (HO-MPBP), 250 μmol of MPBP·HNO_3 were incubated with one litre of CAD58 culture (10^8 cells/mL, pH 9, 48 h, 30 °C). HO-MPBP was isolated by liquid–liquid extraction and precipitated as its hydrochloride salt. Identity and purity of the product were tested by HPLC with ultraviolet (UV) detection, GC-MS, and ^{1}H-NMR. CAD58 was further characterized regarding the influence of incubation pH (5–10), cell density (10^7–10^8 cells/mL), and incubation time (0–120 h) on metabolite formation using the substrates dextromethorphan and MPBP. The preparative experiment yielded 40 mg (141 μmol) of HO-MPBP·HCl with a purity of >98%. In the characterization experiments, the metabolite formation rate peaked at pH 8. A linear relationship was observed between cell density and metabolite formation ($R^2 > 0.996$). The rate of metabolite formation was slower in the earlier stages of incubation but then increased. For HO-MPBP, it became constant in the time interval of 2.5–34 h ($R^2 > 998$).

1. Introduction

Reference standards of drug metabolites are needed for their structural confirmation and pharmacologic and toxicologic characterization, including studies on their pharmacodynamic and pharmacokinetic properties, on enzyme kinetics of their formation, and on phase II metabolism. However, such metabolite standards are often not commercially available, particularly in the case of new therapeutic drugs or drugs of abuse. The classical chemical synthesis of drug metabolites

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can be cumbersome and stereochemically demanding and hence go beyond the possibilities of most biochemistry or pharmacology/toxicology oriented laboratories. Custom-made metabolite standards are a possible but usually time-consuming and very expensive solution.

Biotechnological synthesis of drug metabolites using cytochrome P450 (CYP) enzymes could be a versatile alternative to classical chemical synthesis and possibly have important advantages over the latter. Firstly, they would yield the metabolites of interest as products, at least if the CYP isozymes responsible for the *in vivo* formation of the respective metabolites were used. Secondly, one would expect only one (major) product when using specific isozymes, because they usually catalyze one metabolic step with high preference or even exclusively. Moreover, the metabolic reactions are generally highly stereoselective. Finally, they can be carried out under mild conditions in comparison to classical chemical reactions that often require high temperatures, high pressure or aggressive and/or toxic chemicals.

The aim of the presented study was evaluating the feasibility of biotechnological synthesis of drug metabolites using human CYP2D6 heterologously expressed in the fission yeast *Schizosaccharomyces pombe* as model enzyme and 1-(4-methylphenyl)-2-pyrrolidin-1-ylbutan-1-one (4′-methyl-α-pyrrolidinobutyrophenone, MPBP) as model substrate. CYP2D6 was chosen as model enzyme, because it is involved in the metabolism of many therapeutic and illicit drugs in humans [1–3]. In addition, CYP2D6 and metabolic steps catalyzed by this isozyme are of particular interest in pharmacology and toxicology because CYP2D6 is polymorphically expressed [3]. In CYP2D6 poor metabolizers, the metabolic clearance of drugs predominantly metabolized by CYP2D6 can be dramatically reduced increasing the risk of side effects and drug toxicity. Fission yeast, which was chosen for heterologous expression of human CYP2D6, was first isolated by Lindner from East African millet beer in 1893 [4]. The species name is derived from the Swahili word for beer ''pombe''. It is an ancient species diverged from bakers yeast 400 million years ago. This organism was chosen for expression of human CYP2D6 for various reasons. It is a eukaryote with structures and processes that resemble the ones encountered in higher eukaryotes [5–9]. Its genome has been fully sequenced [10] and genetic techniques for working with this species are well established [11]. Moreover, fission yeast has only two putative endogenous CYPs by homology [10] decreasing the risk for unwanted side reactions. Finally, it has already been successfully used for expression of human CYPs [12–16]. The model drug MPBP is a new designer drug. Together with α–pyrrolidinopropiophenone (PPP) [17,18], 4′-methyl-α-pyrrolidinopropiophenone (MPPP) [17–19], 4′-methyl-α-pyrrolidinohexanophenone (MPHP) [20], 4′-methoxy-α-pyrrolidinopropiophenone (MOPPP) [21], 3′,4′-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP) [17,22], and α–pyrrolidinovalerophenone (PVP) it belongs to the class of pyrrolidinophenone-type designer drugs, some of which are scheduled as controlled substances in Germany. Their chemical structures are given in Fig. 1. MPBP was seized as powder by the German police. It is assumed to be taken orally as the other pyrrolidinophenones, which are distributed among drug abusers as tablets, capsules, or powders [17]. Statements on the frequency of occurrence of the pyrrolidinophenones cannot be made, because they cannot be detected with usual routine analysis procedures [18–23] and might, therefore, have been overlooked. So far, little information about the dosage of as well as the pharmacologic and toxicologic effects of the pyrrolidinophenones is available. However, they may be expected to be very similar to those of pyrovalerone (4′-methyl-α-pyrrolidinovalerophenone), a potent psychostimulant, because of their close structural relation to this drug as shown in Fig. 1. Concerning its mechanism of action, it has been reported that pyrovalerone releases dopamine and norepinephrine from respective nerve terminals [24,25] and inhibits the reuptake of these neurotransmitters [26]. In comparison to amphetamine, it has been reported to have similar psychostimulant effects but less influence on motor function in animals and humans [27–29]. Pyrovalerone had been studied as a therapeutic drug [30–32], but was withdrawn from the market and scheduled as a controlled substance after a report of its intravenous abuse [33]. A similar pharmacological profile of the pyrrolidinophe-

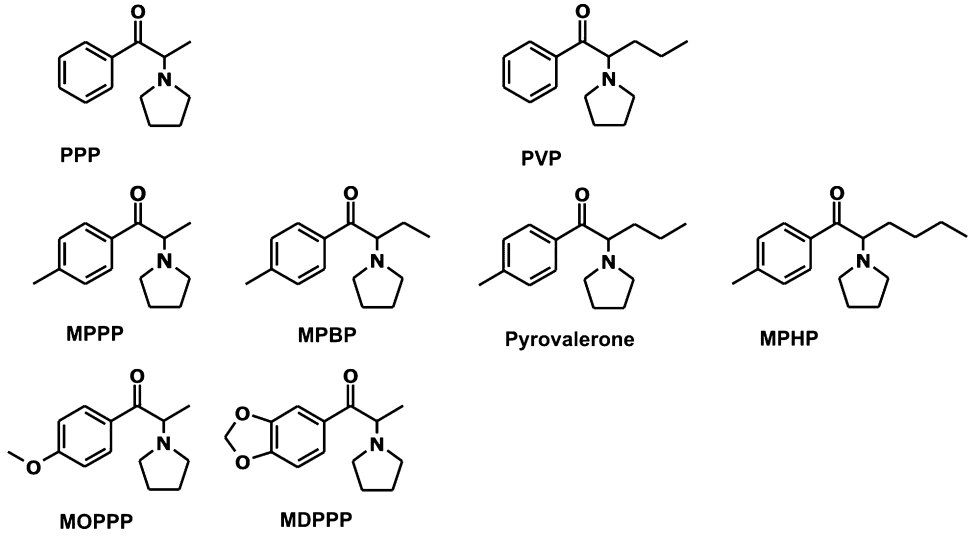

Fig. 1 – Chemical structures of pyrrolidinophenone-type designer drugs and of the psychostimulant pyrovalerone.

nones would clearly be in line with their abuse as stimulant designer drugs. The qualitative metabolism of MPBP has been studied in rats and 4′-methyl hydroxylation followed by oxidation to the respective carboxylic acid was found to be the main metabolic pathway [23]. This is in line with the metabolism of other 4′-methyl pyrrolidinophenones in animals [18,20,23,34–37] and humans [34]. An initial screening study in the authors' laboratory using insect cell microsomes with cDNA expressed human CYPs showed that CYP2D6, CYP2C19, and CYP1A2 were involved in the 4′-methyl hydroxylation of MPBP. For further studies on the contribution of each of these isozymes the reference standard of the resulting metabolite 4′-hydroxymethyl-α-pyrrolidinobutyrophenone (HO-MPBP) was needed. This is why biotechnological semi-preparative synthesis of HO-MPBP from MPBP was chosen as the model reaction in the present paper.

2. Methods and materials

2.1. Chemicals and reagents

MPBP-HNO_3 was provided from Hessisches Landeskriminalamt (Wiesbaden, Germany) for research purposes. The purity and identity had been proven by mass spectrometry, infrared and ^{1}H-NMR spectroscopy [38]. *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Fluka (Steinheim, Germany). Hydrobromide of dextromethorphan (DXM) and tartrate of dextrorphan (DXOH) were from MP Biomedicals. All other chemicals and biochemicals used were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

2.2. Construction of fission yeast strain CAD58

2.2.1. CYP2D6 cDNA and vectors and parental strain

The human CYP2D6 cDNA sequence was retrieved from UniProtKB/Swiss-Prot (entry P10635), optimized for fission yeast codon usage by Entelechon GmbH (Regensburg, Germany) and delivered as a pPCR-ScriptAmpSK(+) (Stratagene, La Jolla, CA, USA) clone. The CYP2D6 cDNA was synthesized with 5′ terminal NdeI and 3′ terminal BamHI restriction sites. Expression vector pCAD1 was already described [12].

2.2.2. Strains and media

Fission yeast strain NCYC2036 with genotype *h*$^-$ *ura4.dl18* was used as parental strain. Biomass was produced in batch using Edinburgh minimal media (EMM) with necessary supplements according to the genotype of the particular strain. Permanent cultures were stored in double concentrated yeast extract with additives [2X YEA; 6 g glucose, 1 g yeast extract (Difco®), 20 mg of each lysine, histidine, adenine, uracil and leucine dissolved in a 75 mL water/25 mL glycerol mixture].

2.2.3. Strain construction

The CYP2D6 cDNA was cut out from pPCR-ScriptAmpSK(+) with NdeI/BamHI and ligated with NdeI/BamHI restricted pCAD1 to yield pCAD1-HsaCYP2D6 using standard molecular biology techniques. Afterwards, pCAD1-HsaCYP2D6 was restricted with NotI and separated from the bacterial vector backbone by agarose gel electrophoresis. The precipitated NotI integration fragment was dissolved in water and directly used for NCYC2036 transformation according to the protocol for cryopreserved, competent fission yeast cells [39]. Transformants were streaked on EMM + leucine + thiamine dishes and grown for approximately 72 h yielding colonies of the new strain CAD58. Cells of CAD58 were aliquoted and stored at −80 °C in 2X YEA for further testing.

2.2.4. Biomass production

Cells of strain CAD58 from permanent cultures were streaked on EMM dishes containing 0.01% leucine (w/v) and 5 μmol/L thiamine and grown for three days at 30 °C. Cell material from a plate was transferred to a 10 mL EMM preculture with 0.01% leucine (w/v) in absence of thiamine. From now on, all subsequent cultures were done in absence of thiamine to keep the *nmt1* promoter in an active state. Biomass production was scaled up by factor 10 to yield 100 mL or 1 L cell suspensions in batch cultures. Usually, the final cell density was around 5×10^7 cells/mL with cells being in the stationary growth phase. After biomass production, the cells were centrifuged at 3000 *g* for 5 min except for 1 L cultures where centrifugation was carried out at 5000 *g* for 25 min. The biomass was washed three times with cold, deionized water and finally resuspended in certain volumes of 100 mmol/L NaH_2PO_4/Na_2HPO_4 at a certain pH as required for the respective experimental conditions.

2.2.5. Test for dextromethorphan-O-demethylase activity

Activity of expressed human CYP2D6 was tested by *O*-demethylation of DXM. For this purpose, DXM (1 mmol/L) was incubated with CAD58 or NCYC2036 cells suspended to 10^8 cells/mL in 10 mL of 100 mmol/L NaH_2PO_4/Na_2HPO_4 buffer (pH 9) containing 2% glucose (v/v). Incubation was carried out in 250 mL Erlenmeyer flasks with a cellulose stopper at a shaking velocity of 150 rpm. Incubation time and temperature were 72 h and 30 °C, respectively. After incubation, the cells were separated from the supernatants by centrifugation at 5000 *g* for 20 min and 800 μL of supernatant were transferred to 1.5 mL polypropylene reaction caps. After adding 250 μL of a mixture of 37% hydrochloric acid-2.3 mol/L ammonium sulfate-10 mol/L sodium hydroxide (1:2.5:1.5 v/v/v) and 500 μL of extraction solvent (dichloromethane-isopropanol-ethyl acetate, 1:1:3 v/v/v), the reaction caps were sealed and left on a rotary shaker for 1 min. After phase separation by centrifugation (10,000 *g*; 2 min), the organic phases (upper) were transferred to autosampler vials and evaporated to dryness under a stream of nitrogen at 56 °C. The dry residues were acetylated with 100 μL of acetic anhydride-pyridine (3:2, v/v) for 5 min under microwave irradiation at 440 W. The excess reagent was evaporated and the residues were reconstituted in 100 μL of methanol. Aliquots (2 μL) of the acetylated extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) as described below.

2.3. Biotechnological synthesis and isolation of HO-MPBP

Seventy-five milligrams (250 μmol) of MPBP-HNO_3 were incubated (30 °C, 48 h) in 1 L of 100 mmol/L phosphate buffer (pH 9) containing 2% glucose (m/v) and 10^8 CAD58 cells/mL.

Thereafter, the cells were separated from the supernatants by centrifugation at 5000 *g* for 20 min. The supernatant was basified with 3 mL of 10 mol/L NaOH and extracted with an equal volume of ethyl acetate. The organic phase was evaporated to dryness and the aqueous phase was discarded. The dry extract was reconstituted in 20 mL of ethyl acetate and extracted with 10 mL of 0.01 mol/L HCl. After extraction, the phases were separated by centrifugation and the organic phase was discarded. The aqueous phase was basified with 1 mL of 10 mol/L NaOH and twice extracted with 20 mL of ethyl acetate. After drying over anhydrous sodium sulfate, the organic phase was evaporated to a volume of approximately 10 mL. Then, one drop of 10 mol/L HCl was added and after vigorous shaking for 1 min, the mixture was left at room temperature for 4 h and subsequently centrifuged (1500 *g*, 2 min). The supernatant was removed and the precipitated product was twice washed with 1 mL of ethyl acetate. Finally, the product was dried at 75 °C under reduced pressure.

Approximately 1 mg of product was dissolved in methanol. For identity and purity check by GC-MS, 10 μL of this solution were transferred to an autosampler vial and evaporated to dryness under a stream of nitrogen at 56 °C. The residue was reconstituted in 50 μl of ethyl acetate. After adding 50 μL of MSTFA the vial was sealed and derivatization was carried out for 5 min under microwave irradiation at 440 W. A 3 μL aliquot was injected into the GC-MS system with an alcohol- and water-free syringe. For structural confirmation by proton nuclear magnetic resonance (^{1}H-NMR), solutions of MBPB·HNO_3 and of the product were prepared in D_2O (10 mg/mL). ^{1}H NMR spectra (500 MHz) were recorded on a Bruker DRX 500 at 298 K. The chemical shifts were given in δ values (ppm) relative to the solvent peak (HDO) at δ_H 4.80. For purity check by high-performance liquid chromatography (HPLC), 100 μL of the above-mentioned methanolic solution was transferred to an autosampler vial and evaporated to dryness under a stream of nitrogen at 56 °C. The residue was reconstituted in 100 μl of mobile phase and 20 μL were injected into the HPLC system.

2.4. Characterization of S. pombe strain CAD58

2.4.1. Studies on the influence of pH on metabolite formation

MPBP or DXM (1 mmol/L) were incubated with CAD58 (5×10^7 cells/mL) in 1 mL of 100 mmol/L phosphate buffer containing 2% glucose (v/v). The pH values of the phosphate buffers were 5.0, 6.0, 7.0, 8.0, 8.5, and 9.5. Incubation was carried out in deep well (2 mL) microtiter plates (MTP) at a shaking velocity of 450 rpm. Incubation time and temperature were 4 h and 30 °C, respectively. After incubation, the cells were separated from the supernatants by centrifugation at 5000 *g* for 25 min and the supernatants frozen and stored at −20 °C until analysis. The concentrations of HO-MPBP and DXOH in the supernatants were determined by HPLC analysis and plotted versus the respective pH values. All incubations were performed in duplicate.

2.4.2. Studies on the influence of cell density on metabolite formation

MPBP or DXM (1 mmol/L) were incubated with CAD58 in 1 mL of 100 mmol/L phosphate buffer (pH 8) containing 2% glucose (v/v). The cell densities in the incubation mixture were 10^7, 5×10^7, and 10^8 cells/mL. Incubations were carried out in deep well MTPs at a shaking velocity of 450 rpm. Incubation time and temperature were 4 h and 30 °C, respectively. After incubation, the cells were separated from the supernatants by centrifugation at 5000 *g* for 25 min and the supernatants frozen and stored at −20 °C until analysis. The concentrations of HO-MPBP in the supernatants were determined by HPLC analysis and plotted versus the respective cell densities. All incubations were performed in duplicate.

2.4.3. Studies on influence of incubation time on metabolite formation

MPBP or DXM (1 mmol/L) were incubated with CAD58 (10^8 cells/mL) in 1 mL of 100 mmol/L phosphate buffer (pH 8) containing 2% glucose (v/v). Incubation was carried out in deep well MTPs at a shaking velocity of 450 rpm. Incubation times were 0, 2, 4, 6, and 8 h. Incubation temperature was 30 °C. After incubation, the cells were separated from the supernatants by centrifugation at 5000 *g* for 25 min and the supernatants frozen and stored at −20 °C until analysis. The concentrations of HO-MPBP and DXOH in the supernatants were determined by HPLC analysis and plotted versus the respective incubation times. All incubations were performed in duplicate.

2.4.4. Long-term experiments on time-courses of metabolite formation

MPBP or DXM (1 mmol/L) were incubated with CAD58 (10^8 cells/mL) in 1 L of 100 mmol/L phosphate buffer (pH 8) containing 2% glucose (v/v). Incubation was carried out in 2 L Erlenmeyer flasks at a shaking velocity of 150 rpm. Incubation time and temperature were 120 h and 30 °C, respectively. Samples (1 mL) were drawn at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10, 12, 22, 26, 30, 34, 48, 55, 58, 81, 85.5, 100, 105.5, and 120 h. The cells were separated from the supernatants by centrifugation at 5000 *g* for 25 min and the supernatants frozen and stored at −20 °C until analysis. The concentrations of HO-MPBP and DXOH in the supernatants were determined by HPLC analysis.

2.5. Analytical methods

2.5.1. GC-MS analysis of substrates and metabolites

The acetylated or trimethylsilylated sample extracts were analyzed using a Hewlett Packard (HP, Agilent Technologies, Waldbronn, Germany) HP 6890 Series GC system combined with an HP 5972 Series mass selective detector, an HP 6890 Series injector and an HP Chem Station G1701AA version A.03.00. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m × 0.2 mm I.D.), cross linked methylsilicone, 330 nm film thickness; injection port temperature, 280 °C; carrier gas, helium; flow-rate 1 mL/min; column temperature, programmed from 100 to 310 °C at 30 °C/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode, *m/z* 50-550 u; EI ionization mode: ionization energy, 70 eV, ion source temperature, 220 °C; capillary direct interface heated at 260 °C. DXM, (acetylated) DXOH, MPBP and (trimethylsilylated) HO-MPBP were identified by comparing the mass spectra in the respective samples to the reference mass spectra [23,40].

2.5.2. *High performance liquid chromatography (HPLC)*

A HP 1050 series HPLC system consisting of a quaternary pump, a degasser, an autosampler, a variable wavelength (VWD) detector and an HP 1046A fluorescence detector was used. The stationary phase was a Zorbax® 300-SCX column (2.1 × 150 mm, 5 μm). The mobile phase A consisted of 5 mmol/L ammonium formate buffer brought to pH 3 with formic acid. Mobile phase B consisted of acetonitrile containing 1% (v/v) formic acid.

MPBP and HO-MPBP were analyzed using an injection volume of 10 μL of untreated incubation supernatant, a mobile phase composition of A:B 55:45 (v/v), a flow rate of 0.9 mL/min, ultraviolet (UV) detection at λ = 265 nm, and a run time of 8 min. DXM and DXOH were analyzed using an injection volume of 10 μL of untreated incubation supernatant, a mobile phase composition of A:B 45:55 (v/v), a flow rate of 1.0 mL/min, and fluorescence detection with $\lambda_{excitation}$ = 280 nm and $\lambda_{emission}$ = 310 nm.

Concentrations of HO-MPBP and DXOH in the supernatants were determined using calibration curves in the range of 1–800 μmol/L of the respective analyte. Absolute peak areas of the calibrators were plotted against the respective analyte concentrations and a linear least squares regression model was used to calculate the calibration line.

3. Results

3.1. *Construction of S. pombe strain CAD58*

Growth and phenotype of CAD58 cells were similar to those of the parental strain. Functional expression of human CYP2D6 in CAD58 was tested by incubation experiments with DXM. In subsequent GC-MS analysis, DXOH was detected in the extracts of supernatants from CAD58 incubations but not in those of the wild-type strain NCYC2036.

3.2. *Biotechnological synthesis and isolation of HO-MPBP*

CAD58 was used for biotechnological synthesis of the MPBP metabolite HO-MPBP. HPLC analysis of the incubation supernatants showed that approximately 250 μmol (75 mg) of MPBP was virtually completely metabolized after 48 h with only very small amounts of parent drug detectable. This is illustrated in Fig. 2 (bottom) that shows chromatograms of supernatants of incubation mixtures before (dotted line) and after 45 h incubation (solid line) of MPBP with CAD58. After extraction of the product followed by precipitation with hydrochloric acid, a white, crystalline powder was obtained with a yield of approximately 40 mg (141 μmol) or 56% of the theoretical maximum yield.

GC-MS analysis showed that the EI mass spectrum and the retention index of the trimethylsilyl derivative of the product were identical with those of the postulated metabolite HO-MPBP given in reference [23]. In Fig. 3, the ^{1}H–NMR spectra of the parent drug (upper panel) and the product (lower panel) are shown. As indicated by the arrow, the singlet (a) at 2.45 ppm, which corresponds to the three protons of 4′-methyl group of MPBP [38], has disappeared in the spectrum of the product while a new singlet signal corresponding to two

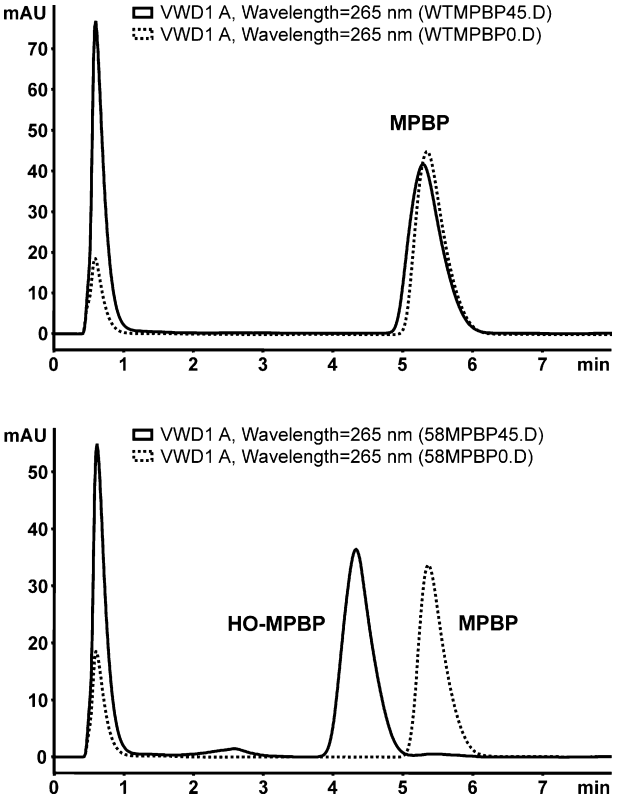

Fig. 2 – Chromatograms (λ = 265 nm) of incubation mixtures (100 mmol/L phosphate buffer) with 250 μM MPBP and 10^8 cells/mL of NCYC2036 (top) or CAD 58 (bottom). Dotted lines represent samples taken before incubation. Solid lines represent samples taken after 45 h of incubation at 30 °C.

protons has appeared at 4.77 ppm right next to the solvent signal (see inset). These observations are in exact agreement with hydroxylation at the 4′-methyl group and hence confirm the postulated structure of HO-MPBP. The purity of the product was studied by HPLC, GC-MS and ^{1}H-NMR and was found to be very high. While traces of parent drug were detected with all three methods no other impurities were detected. In HPLC analysis, the peak area of the parent drug accounted for approximately 1.5% of total peak area (parent drug + metabolite). Hence, assuming similar absorption coefficients of parent drug and metabolite, the purity of the product was 98.5%.

3.3. *Characterization of S. pombe strain CAD58*

The results of the experiments on the influence of incubation pH on metabolite formation are shown in Fig. 4, where HO-MPBP and DXOH concentrations in incubation supernatants have been plotted against the initial pH of the incubation mixture. In both cases metabolite concentrations steeply increased up to pH 8 and then slightly dropped again at higher pH values.

In the experiments on the influence of cell density on metabolite formation, a linear relationship was found between HO-MPBP (R^2 = 0.999) and DXOH (R^2 = 0.996) forma-

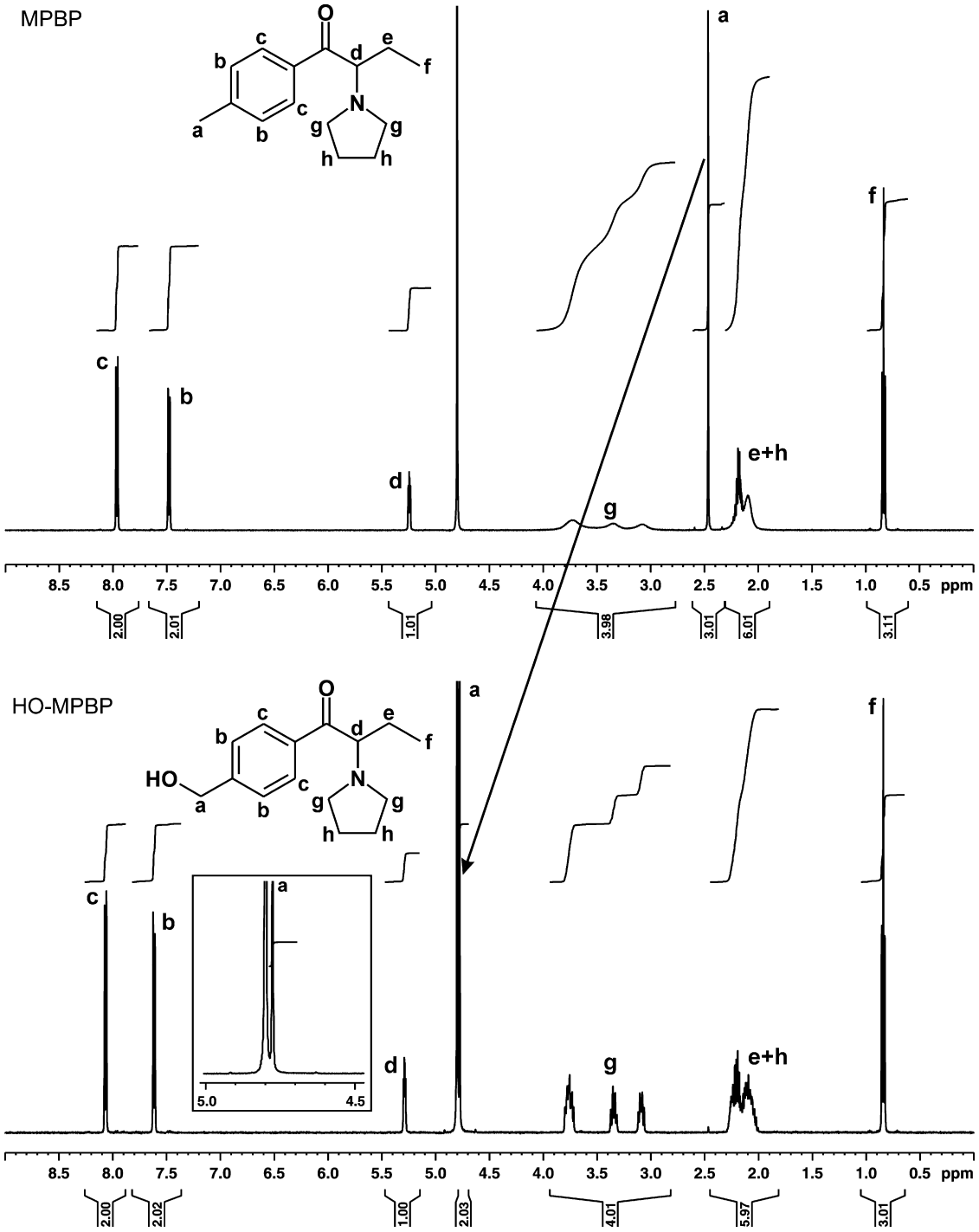

Fig. 3 – ^{1}H-NMR spectra of MPBP (upper panel) and HO-MPBP (lower panel). The labeling of the positions of the chemical structures corresponds to the labeling of proton signals in the respective panels. The arrow indicates the shift of 4′-proton signals (a) caused by hydroxylation of the 4′-methyl group. The inset shows an enlargement of the area between 4.50 and 5.00 ppm for better resolution of the shifted proton signal (4.77 ppm) from the solvent signal (4.80 ppm).

tion and cell density over a range of one order of magnitude, that is from 10^{7} to 10^{8} cells/mL. In the short-term (0 to 8 h with 2 h sampling intervals) experiment on time-dependence of metabolite formation, metabolite formation seemed to start slow and then increased, finally reaching a seemingly linear relationship between 4 and 8 h. The results of the long-term experiment (120 h) with more frequent sampling times are given in Fig. 5, in which the concentrations of HO-MPBP (upper panel) and DXOH (lower panel) have been plotted versus incubation time. The time courses for both analytes could be divided into three phases. Phase 1 ranged from 0 to 2.5 h for both HO-MPBP and DXOH. It was characterized by a steady increase of the rate of metabolite formation. In phase 2, metabolite formation became constant leading to a linear

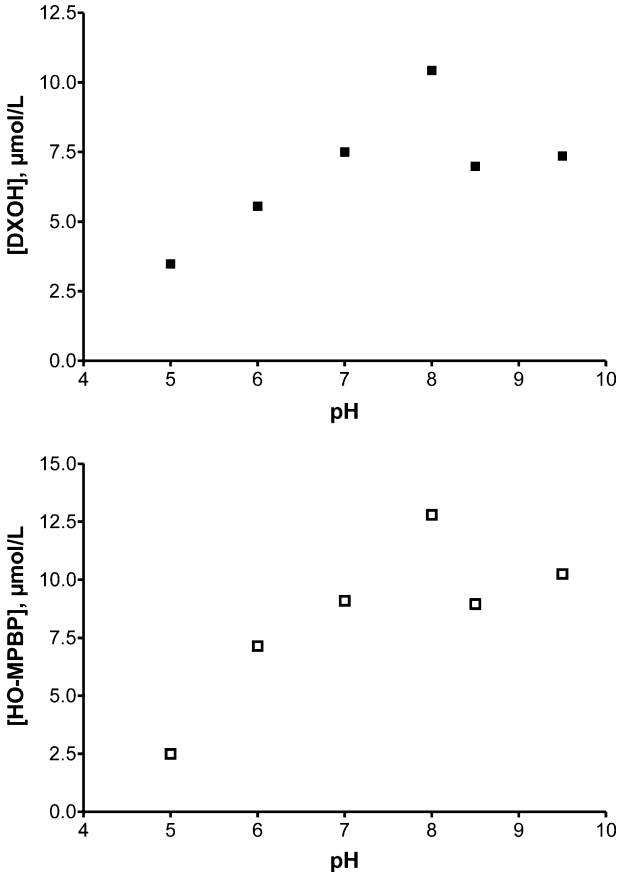

Fig. 4 – Plots of DXOH (upper panel) and HO-MPBP (lower panel) concentrations versus incubation pH. Incubations were performed with 1 mmol/L MPBP or DXM and 5×10^7 cells/mL CAD 58 in 100 mmol/L phosphate buffer for 4 h at 30 °C.

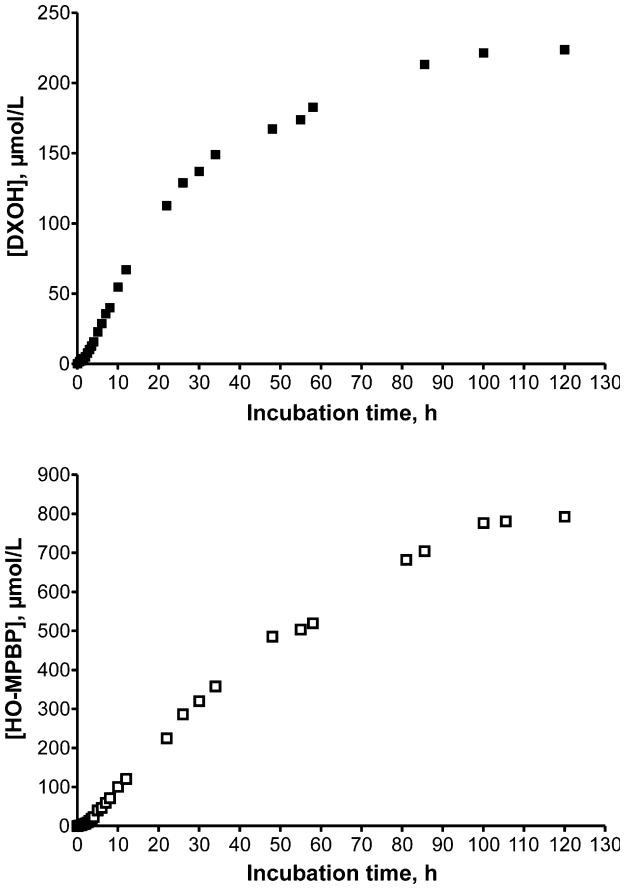

Fig. 5 – Plots of DXOH (upper panel) and HO-MPBP (lower panel) concentrations versus incubation time. Incubations were performed with 1 mmol/L MPBP or DXM and 10^8 cells/mL CAD 58 in 100 mmol/L phosphate buffer for 120 h at 30 °C.

increase of metabolite concentrations in the incubation supernatants. For HO-MPBP this phase ranged from 2.5 to 34 h. The formation rate estimated by linear regression ($R^2 = 0.998$) was 11.3 ± 0.14 μmol/h or 1.9 pmol/min/10^6 cells. For DXOH, phase 2 was shorter ranging from 2.5 to 12 h and was characterized by a formation rate of 6.5 ± 0.08 μmol/h or 1.1 pmol/min/10^6 cells ($R^2 = 0.999$). Finally, phase 3 was characterized by formation rates steadily decreasing to almost zero after 120 h. The results of pH measurements in the supernatants of the long-term experiment showed that the pH values steeply decreased from pH 8 at the start of the incubation to approximately pH 7.4 after 2.5 h, remained constant for the next 2.5 h, and then slowly and steadily decreased to pH 6 after 48 h of incubation. Thereafter, a slight increase to pH 7.3 at 120 h was observed.

4. Discussion

The aim of the present study was evaluating the feasibility of biotechnological synthesis of drug metabolites using human CYP2D6 heterologously expressed in the fission yeast *Schizosaccharomyces pombe* as model enzyme and MPBP as model substrate. After transformation, fission yeast strain CAD58 constructed for this purpose had acquired the capability to catalyze the CYP2D6 specific DXM O-demethylation absent in the wild-type strain. This proved expression of functional CYP2D6 enzymes in CAD58. A similar observation was made in the case of MPBP. CAD58 (Fig. 2, bottom) but not the wild-type strain (Fig. 2, top) was able to catalyze 4′-methyl hydroxylation of MPBP.

After some preliminary initial experiments on pH dependency of MPBP metabolism and expected maximum turnover rates, the experiment for biotechnological synthesis of HO-MPBP was carried out with approximately 250 μmol (75 mg) MPBP-HNO_3 in 1 L of CAD58 culture (10^8 cells/mL) at an initial pH of 9. Almost complete metabolism of MPBP to HO-MPBP after 48 h eliminated the need for chromatographic separation of parent drug and metabolite. The product was isolated from supernatants obtained after separation of the CAD58 cells by centrifugation rather than the entire incubation mixture, because the supernatants contained only few matrix compounds. Denaturing of the cells to release intracellular HO-MPBP would also have released many unwanted intracellular compounds. Moreover, the cells accounted for only a few percent of the total volume of the incubation mixture, so the

loss of product due to the centrifugation step was probably minimal. Workup of the rather clean supernatants allowed using a simple classical back extraction approach. The total yield of about 56% of the theoretical maximum yield of HO-MPBP may seem rather low, but it must be considered that a generic rather than an optimized isolation procedure had been used. Moreover, the amount of product was sufficient for further experiments to characterize CAD58 and for studies on the contribution of CYP2D6 and other CYP isozymes to MPBP 4′-hydroxylation which will be described elsewhere.

In order to further characterize CAD58 and its metabolic CYP2D6 activity, a number of experiments were performed to study the influence of various parameters on metabolite formation and the *in vivo* enzyme kinetics of the heterologously expressed CYP2D6 enzymes. DXM was used as a substrate, because it is a well-established test substrate for CYP2D6. MPBP was used because it was the model substrate for biotechnological synthesis of metabolites in the present study. In the first preliminary experiments, incubations with CAD58 had been performed with non-buffered yeast cultures, which had a pH of approximately 3. Only very little metabolite formation was observed under these conditions. This can be explained by the basic properties of the tested substrates DXM and MPBP. At pH 3, these compounds are present in the protonated forms, which are too hydrophilic to cross the lipophilic cell membrane. However, the substrates must first cross the cell membrane to gain access to the endoplasmic reticulum localized CYP2D6 enzyme by which they can then be metabolized. While substrate diffusion into the cells could be expected to increase with rising pH values, it was initially not clear which pH values would still be tolerated by *S. pombe* which usually grows under acidic conditions. The fact that maximum metabolite formation was observed at pH 8 shows that CAD58 cells tolerated high pH values very well, which was quite surprising considering that this was three pH units above the pH of EMM. However, this finding is very important for future research with CYP isozymes heterologously expressed in *S. pombe*, because it shows that the pH conditions can be adapted to the chemical properties of the substrates rather than the biological system. Based on the described findings, pH 8 was chosen for all further incubation experiments. A very strong linear correlation was found between metabolite formation and cell density over a range of 10^7 to 10^8 cells/mL, so the latter cell density was used in all further incubation experiments. Cell densities higher than 10^8 cells/mL were not evaluated in the present study. However, preliminary experiments with other substrates have meanwhile indicated that formation rates further increase with cell densities above 10^8 cells/mL, but it is not clear whether this increase is still linear.

The short-term experiment on time-dependence of metabolite formation proved to be too short and the sampling intervals too long to draw definitive conclusions, so a long-term experiment with frequent sampling was designed. The three phases observed in this experiment may be interpreted as follows. At the beginning of the incubation, the substrate concentrations are very high in the incubation medium, but at this time there is no substrate inside the cells, i.e. at the site of the enzyme. During the early phase of incubation (phase 1), the substrate penetrates into the cells by diffusion driven by the gradient between extra-cellular and intra-cellular substrate concentrations. Thus, the substrate concentrations at the enzyme and consequently the rates of metabolite formation increase. The constant rates in phase 2 might be explained by equilibrium between the amount of substrate entering the cell by diffusion and the amount of substrate metabolized. Alternatively, constant rates would be expected when the intracellular substrate concentrations have become sufficiently high to saturate the enzyme. The decrease of formation rates in phase 3 is more difficult to explain. Decreasing catalytic activities of CAD58 cells during long-term incubation could explain the phenomenon but would be in contrast to the observation that HO-MPBP formation rates remained stable until 34 h while those for DXOH already started decreasing after 12 h. Another possible explanation would be decreasing amounts of substrate diffusing into the cells because of decreasing extra-cellular concentrations. For MPBP, this might indeed be the reason, because extra-cellular substrate concentrations had already dropped by half when the formation rate of HO-MPBP started decreasing. However, it seems quite unlikely, that this is the explanation in the case of DXM, where substrate concentrations were still very high when the DXOH formation rates started to decline. Another potential reason for decreasing substrate diffusion into the cells could be a decreasing pH of the incubation mixture. In fact, such a decrease of pH was observed. This was not surprising because *S. pombe* cells prefer acidic conditions and hence try to acidify the basic incubation medium by the action of a plasma membrane H^+-ATPase [41]. Considering that the incubation mixture was buffered, it is actually quite remarkable that a minimum pH of 6 was reached during incubation. However, the steepest decrease of pH was observed between 0 and 2.5 h, a time interval where metabolite formation actually increased. Moreover, the steady pH decrease from 7.4 to 6 showed a large (DXM) or even complete (MPBP) overlap with the linear phases of metabolite formation. In fact, the formation of HO-MPBP only started to decrease when pH values had started to increase again. For these reasons, pH changes are an unlikely explanation for phase 3. Finally, decreasing formation rates in phase 3 might be explained by product inhibition caused by metabolites accumulating inside the cells. From the existing data, it cannot be concluded whether one or more of these hypotheses are true. Further studies performed under controlled fermentation conditions rather than an open batch approach as described here would be necessary to acquire more information about the long-term viability of CAD58 cells and transport processes into and out of the cells.

While the results of the present study indicate that long-term incubation over several days can be beneficial with respect to metabolite yields, this may not always be the case. If a CYP isozyme is capable of catalyzing more than one metabolic reaction of the same substrate, long incubation times may lead to side-products or even secondary metabolites, i.e. metabolites of metabolites, and hence decrease the yield of the target product. On the other hand, if one is interested in targeting a secondary metabolite, even longer incubation times than used in the present study might be helpful.

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