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Isolation and Purification of the 4'-Hydroxymethyl Metabolites of the Designer Drugs 4'-Methyl-α-pyrrolidinopropiophenone and 4'-Methyl-α-pyrrolidinohexanophenone Biotechnologically Synthesized using Fission Yeast Co-expressing Human Cytochrome P450 Reductase and Human CYP P450 Isozymes 2D6 or 2C19

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Meiner Familie.

Table of contents

ZUSAMMENFASSUNG

In den letzten Jahren wurden wiederholt Vertreter einer neuen Klasse von psychostimulierenden Designerdrogen, den so genannten Pyrrolidinophenonen, sichergestellt. Dies ist als Teil eines Trends anzusehen durch gezielte chemische Abwandlung von Betäubungsmitteln oder deren Leitstrukturen neue Designerdrogen zu synthetisieren, die nicht mehr der Betäubungsmittelgesetzgebung unterliegen. Solche neuen Designerdrogen sind in der Regel nicht auf mögliche Nebenwirkungen oder Toxizität untersucht und bergen daher ein unbekanntes Gefährdungspotential für die Konsumenten. Um dieses abschätzen zu können, sind Untersuchungen zum Stoffwechsel dieser neuen Drogen erforderlich, wofür Referenzsubstanzen der gebildeten Hauptmetaboliten benötigt werden.

Das Ziel der vorliegenden Arbeit war die biotechnologische Synthese, die Isolierung und Aufreinigung von Stoffwechselprodukten zweier Pyrrolidinophenone. Die 4'-Hydroxymethyl-Metaboliten der Designerdrogen 4'-Methyl-α-pyrrolidinopropiophenon und 4'-Methyl-α-pyrrolidinohexanophenon wurden unter Verwendung der Spalthefestämme CAD64, welcher humane Cytochrom-P450-Reduktase und das humane Cytochrom P450 Isozym CYP2D6 exprimiert, und CAD66, welcher humane Cytochrom-P450-Reduktase und humanes CYP2C19 exprimiert, synthetisiert. Nach Inkubation mit den entsprechenden Muttersubstanzen konnten die entstandenen Produkte, 4'-Hydroxymethyl-α-pyrrolidinopropiophenon und 4'-Hydroxymethyl-α-pyrrolidinohexanophenon aus den jeweiligen Hefekulturen durch Festphasenextraktion isoliert, mittels semipräparativer Hochleistungsflüssigkeitschromatographie von verbleibender Muttersubstanz und Matrixbestandteilen getrennt, durch eine Flüssig-flüssig-Extraktion aus dem Fließmittel extrahiert und in Form ihrer Hydrochloride ausgefällt werden. Die Identität der synthetisierten Produkte konnte mittels Gaschromatographie-Massenspektrometrie und Magnetresonanz-Spektroskopie bestätigt werden. Zudem ergab die Analyse mittels Hochleistungsflüssigkeitschromatographie mit UV-Detektion eine hohe Reinheit der Produkte.

In dieser Arbeit konnte gezeigt werden, dass die biotechnologische Synthese der beiden Pyrrolidinophenon-Metaboliten eine Alternative zur klassisch-chemischen Mehrstufensynthese darstellt und im Vergleich zu dieser zeitsparender und sicherer im Hinblick auf den geringeren Einsatz gefährlicher Chemikalien ist.

SUMMARY

Over the last years the police repeatedly seized substances belonging to a new class of psychostimulant designer drugs, the so-called pyrrolidinophenones. This can be seen as part of a trend to circumvent the law for controlled substances by specifically changing the chemical structure of certain drugs of abuse. These newly created substances have usually never been studied towards possible side effects or toxicity in humans and are therefore of unknown risk for the consumers. For assessing the latter, studies on the metabolism of these new drugs are necessary for which reference substances of the main metabolites are needed.

The aim of the presented study was the biotechnological synthesis, isolation and clean-up of the metabolites of new designer drugs belonging to the group of pyrrolidinophenones. The 4'-hydroxymethyl metabolites of the designer drugs 4'-methyl-α-pyrrolidinopropiophenone and 4'-methyl-α-pyrrolidinohexanophenone were synthesized using the fission yeast strains CAD64, co-expressing human cytochrome P450 reductase and human cytochrome P450 isozyme CYP2D6, and CAD66, co-expressing human cytochrome P450 reductase and CYP2C19. After incubation with the respective parent drugs, the metabolites, 4'-hydroxymethyl-α-pyrrolidinopropiophenone and 4'-hydroxymethyl-α-pyrrolidinohexanophenone, were isolated from the yeast cultures by solid-phase extraction. Using high-performance liquid chromatography they were then separated from remaining parent drug and matrix compounds to be extracted from the mobile phase by liquid-liquid extraction. Finally, the metabolites could be precipitated as hydrochloric salts. The identity of the synthesized metabolites was confirmed using gas chromatography-mass spectrometry and magnetic resonance spectroscopy. Moreover, high product purity was proven by high-performance liquid chromatography with UV detection.

The present study shows that the biotechnological synthesis of drug metabolites is an alternative to classical chemical multi-step synthesis and in comparison to the latter more time-saving and safer regarding the reduced use of hazardous chemicals and reaction conditions.

1 INTRODUCTION

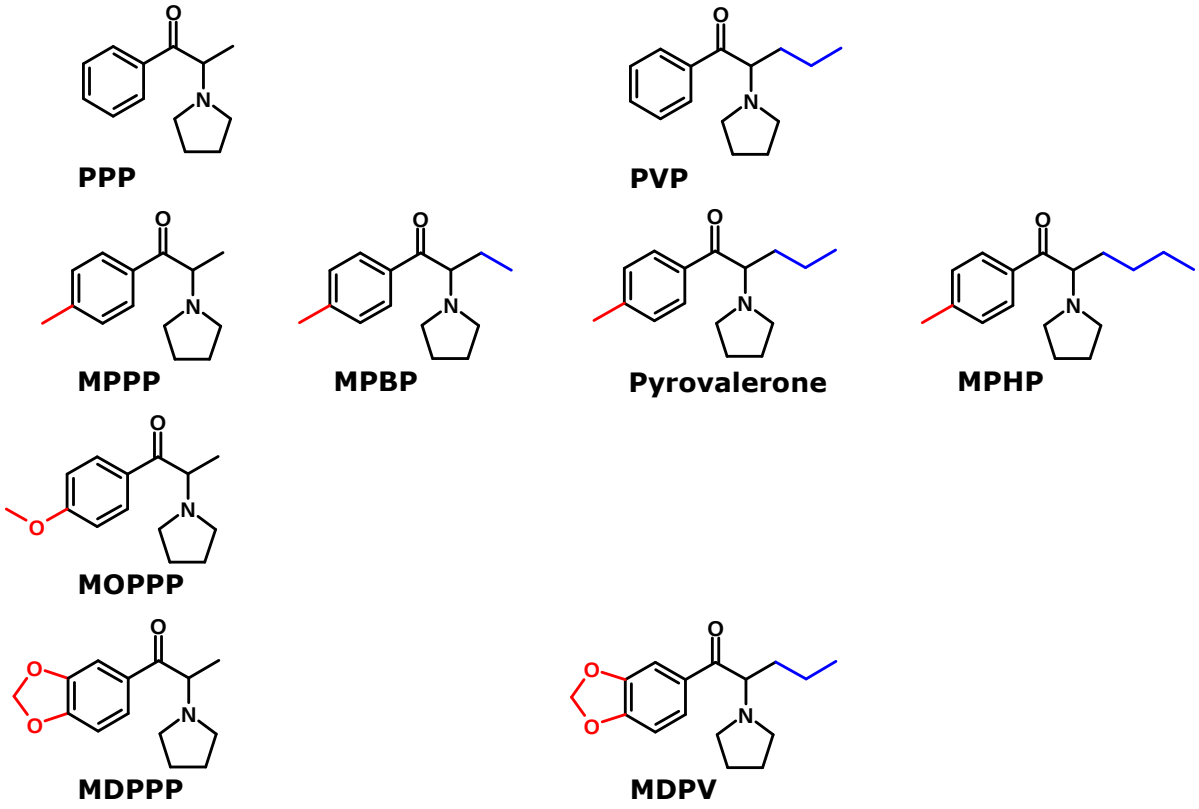
1.1 General aspects of Designer drugs

The appearance of ever new psychoactive compounds on the illicit or grey drug market has become an increasing challenge for authorities and toxicologists in Germany and elsewhere in the world. These substances are usually derived from established drugs of abuse by more or less significant modifications of their chemical structure in illegal private laboratories in order to circumvent the controlled substances laws and/or to optimize the pharmacological properties of the respective drugs. During the last two decades a huge number of new drugs from various drug classes have emerged on the illicit drug market all over the world. Only some of these compounds have meanwhile been scheduled as controlled substances. The majority is still sold as so-called ‘legal highs’ via the internet (Europe) or can even be purchased locally in independent stores (USA) [35,45]. New designer drugs have never been systematically studied towards their potential hazardous effects on the human organism. Hence, there is only little or no information on potential signs of an overdose or side effects of these compounds, especially when they first appear on the market. Also, because of their unknown structure and metabolism there are no standardized methods to prove their presence in biological samples during toxicological screening procedures. Thus, dealing with an intoxicated patient the presence of a new designer drug responsible for possible clinical symptoms presented may be overlooked.

1.2 Pyrrolidinophenones, History and Metabolism

The so-called pyrrolidinophenones are one class of these new designer drugs that have appeared in form of tablets and powders on the illicit drug market in Germany [37]. The chemical structures of the pyrrolidinophenones are schown in Scheme 1. α-Pyrrolidinopropiophenone (PPP) [37,47] is the pyrrolidinophenone-type drug with the simplest structure and all others can be considered as PPP derivatives with an elongated side chain such as α-pyrrolidinovalerophenone (PVP), with a substituted aromatic ring such as 4'-methyl-α-pyrrolidinopropiophenone (MPPP) [37,46,47], 4'-

methoxy-α-pyrrolidinopropiophenone (MOPPP) [48], 3',4'-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP) [37,49], or with an elongated side-chain and a substituted aromatic ring such as 4'-methyl-α-pyrrolidinobutyrophenone (MPBP) [32,55] and 4'-methyl-α-pyrrolidinohexanophenone (MPHP) [51,55]. Most recently, a ring substituted analogue of PVP, namely 3,4-methylenedioxypyrovalerone (MDPV), which is also seen as a synthetic cathinone derivative, has been described in the literature [23,28,31,56]. The route of application of these drugs varies. Oral ingestion, snorting, smoking, and inhalation as well as sublingual, intravenous, intramuscular, or rectal administration have been reported. They have further been taken in combination with other drugs, such as alcohol, beta-blockers, or cannabis to either reduce harmful effects or enhance desired effects [6]. For MDPV, limited prevalence data are available that suggest a considerable popularity of this drug among drug users [23,45]. For all other pyrrolidinophenones, there are no reliable data on the frequency of their abuse which may be due to the fact that the presence of pyrrolidinophenones and their metabolites might have been overlooked in routine drug screenings, because specific analytical methods are required for their detection in urine samples [32,34, 46,47,48,49,51].

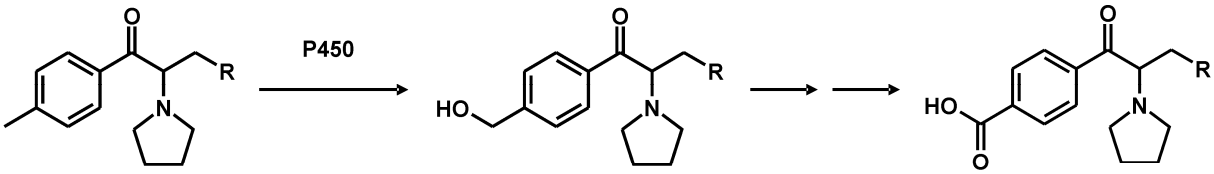


Scheme 1 Chemical structures and acronym names of pyrrolidinophenone-type drugs.

It can be assumed that due to structural similarities the pharmacological properties of the pyrrolidinophenones are similar to the psychostimulant pyrovalerone [4'-methyl-α-pyrrolidinovalerophenone]. This assumption can particularly be made for the 4'-methyl substituted compounds MPPP, MPBP, MPHP and MDPV. Pyrovalerone acts by release [13,40] and reuptake inhibition [27] of dopamine and norepinephrine. The psychostimulant effects of pyrovalerone are similar to those of amphetamine, but pyrovalerone has less influence on the motor system [18,20,52]. Pyrovalerone was marketed as a therapeutic drug in the 1960ies and 1970ies [15,19,44], but was later scheduled as a controlled substance after reports of intravenous abuse [7]. In an internet research performed by *Coppola et al*. MDPV was evaluated towards psychostimulant effects described by drug users. Reported motivations for using MDPV were increased concentration, prolonged sexual performance, increased sociability, energy and euphoria at low doses. At high doses psychostimulant effects were referred to as stronger than those of cocaine or amphetamines [6]. Thus, the assumed similar pharmacologic profile of other pyrrolidinophenone-type designer drugs would certainly explain their abuse potential [34].

Also the reported adverse effects and clinical symptoms can be assumed to be similar within the group of pyrrolidinophenones. For MDPV and MPHP mainly neurological, cardiovascular and psychopathological symptoms have been described, such as: tachycardia, hypertension, hyperthermia, hallucinations, paranoia, aggressive behaviour, liver damage, rhabdomyolysis and consecutive kidney damage, even leading to necessity of intensive care treatment [6,39].

It has been shown in various animal species, that the main metabolic pathway of the 4'-methyl substituted pyrrolidinophenones MPPP [47], MPBP [33], pyrovalerone [25,30,41,42] and MPHP [51] was hydroxylation of the 4'-methyl groups with subsequent oxidation to the respective carboxylic acids as shown in *Scheme 2*. For pyrovalerone, this was also the main metabolic pathway in a human subject [30]. *In vitro* the 4'-hydroxymethyl metabolite of pyrovalerone is also a potent inhibitor of dopamine and norepinephrine transporters [27], although with a lower potency than that of its parent drug. The respective 4'-carboxy metabolite of pyrovalerone was found to be inactive *in vivo* [30]. A recently published case report by *Sauer et al.* of acute poisoning due to MPHP further supports the assumption of similar metabolic pathways as only the 4'-carboxy metabolite was detected in urine samples of the patient [39].



R = H **MPPP**
R = CH_3 **MPBP**
R = C_2H_5 **pyrovalerone**
R = C_3H_7 **MPHP**

Scheme 2 Main metabolic pathway of the 4'-methyl substituted pyrrolidinophenones MPPP, MPBP, pyrovalerone and MPHP. The initial rate limiting step is a P450 catalyzed hydroxylation of the 4'-methyl group that is followed by oxidation of the resulting alcohol to the corresponding carboxylic acid.

In consideration of their structural similarity to pyrovalerone, the 4'-hydroxymethyl metabolites of MPPP, MPBP and MPHP can also be expected to be pharmacologically active. In the main metabolic pathway of these drugs their formation seems to be the rate limiting step. To enable pharmacologic and toxicologic risk assessment of these drugs in human beings it is essential to fully understand their metabolic pathways [34].

1.3 HUMAN CYTOCHROME P450

Cytochrome P450 mono-oxygenases (P450s) were first described in 1958. They were found to be ubiquitous proteins that account for oxidation of numerous compounds, such as steroids, prostaglandins and fatty acids. They are also the major enzymes involved in human drug metabolism for more than 90% of oxidative metabolic reaction of xenobiotics are catalyzed by P450s [16]. So far we know about 57 human P450 isozymes of which 15 have been proven to be responsible for metabolism of xenobiotics. These are P450s 1A1, 1A2, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 3A4, 3A5 and 3A7 [16]. Due to polymorphisms in the genes coding for some CYP450s (e.g. P450s 2C9, 2C19 and 2D6) there is an inter-individual variability of enzyme composition leading to pharmacokinetically distinct subpopulations and subsequently to individual alteration of efficacy and toxicity of drugs [29]. Also there are different drugs and food ingredients which, when co-

ingested, can lead to enzyme inhibition or induction resulting in reduction or enhancement of the activity of P450s. In order to enable realistic risk assessment of increased side effects in poor metabolizers information about the involvement of human P450 enzymes in the 4'-methyl hydroxylation is needed, especially with regard to pharmacogenetic and drug/drug or drug/food interactions.

1.4 SCHIZOSACCHAROMYCES POMBE

Schizosaccharomyces pombe is a fission yeast first isolated in East African millet beer in 1893 by Lindner [24]. The name derives from the Swahili word for beer "pombe". It is an ancient species diverged from baker's yeast around 400 million years ago. *S. pombe* is a eukaryote with structures and processes similar to those encountered in higher eukaryotes [3,14,21,22,38]. Its genome sequence is completely known and genetic techniques for working with this species are well established [1,57]. These are good reasons why *S. pombe* seems to be a suitable organism for heterologous expression of microsomal human P450s involved in drug metabolism. Also fission yeast was found to have only two putative endogenous P450s by homology which significantly decreases the risk for unwanted side reaction leading to biased results [57]. Finally, it has already been described to successfully express mitochondrial human P450s [4,9-11], microsomal human P450s [12, 33, 58] and for biotechnological synthesis of drug metabolites [12,33,34] .

1.5 BIOTECHNOLOGICAL SYNTHESIS OF DRUG METABOLITES

To enable measurement of involvement of human P450s in MPPP and MPHP 4'-methyl-hydroxylation and pharmacologic activity of the resulting metabolites 4'-hydroxymethyl-α-pyrrolidinopropiophenone (HO-MPPP) and 4'-hydroxymethyl-α-pyrrolidinohexanophenone (HO-MPHP), a reference standard of these metabolites is needed. Currently there is no such reference standard commercially available. There have been descriptions of how to chemically synthesize the corresponding metabolite of pyrovalerone using classical chemical synthesis [30]. This would involve eight different steps with an expected overall yield of metabolite of approximately 30%. Studies reported were able to show that biotechnological synthesis of drug metabolites using human P450 isozymes heterologously expressed in *S. pombe* is a

versatile alternative to the rather complicated classical chemical multi-step synthesis [12,33,34]. As model drug MPBP was used and P450 2D6 heterologously expressed in *S. pombe* strain CAD 58 served as model enzyme. By biotechnological synthesis approximately 40 mg of 4'-hydroxymethyl-α-pyrrolidinophenone (HO-MPBP) were obtained with a purity greater than 98%. Following this successful approach new fission yeast strains have been constructed, that heterologously co-express human P450 reductase (hCPR) and either P450 2B6, P450 2C9, P450 2C19, P450 2D6, or P450 3A4 [12,34].

1.6 AIM OF THE STUDY

The aim of the present study was to synthesize the drug metabolites HO-MPPP and HO-MPHP by applying the concept of biotechnological synthesis of drug metabolites using one of the newly constructed *S. pombe* fission yeast strains co-expressing hCPR and human CYP450s.

The following approach was used:

- Incubation of the designer drugs MPPP and MPHP with *S. pombe* strains CAD64 (hCPR + CYP2D6) or CAD66 (hCPR + CYP 2C19) under defined conditions
- Extraction of drug metabolites and remaining parent drug from incubation supernatant using solid-phase extraction (SPE)
- Separation of drug metabolite and remaining parent drug from incubation matrix compounds using semi-preparative high-performance liquid chromatography (HPLC)
- Isolation of drug metabolite from HPLC fractions using liquid-liquid extraction (LLE)
- Precipitation of the purified metabolite products as hydrochloric salts
- Structure confirmation and purity check of the final products by HPLC-UV, gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR).

2 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

MPPP·HCl and MPHP·HNO_3 were provided by the Hessian State Criminal Office (Wiesbaden, Germany) for research purposes. The purity and identity had been proven by mass spectrometry, infrared spectroscopy and ^{1}H-NMR. The *S. pombe* strains CAD64 (co-expressing CYP2D6 and hCPR) and CAD66 (co-expressing CYP2C19 and hCPR) were cloned and characterized by PomBiotech (Saarbrücken, Germany). Varian Bond Elut SCX HF cartridges (5 g, 20 mL) were obtained from Varian (Darmstadt, Germany). *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Fluka (Steinheim, Germany). All other chemicals and biochemicals used were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

2.2 BIOMASS PRODUCTION

Cells from permanent cultures of the respective strains were streaked on Edinburgh minimal media (EMM) dishes containing 0.01 % leucine (w/v) and 5 µM thiamine and grown for 3 days at 30 °C. Cell materi al from the plates was transferred to a 10 mL EMM preculture with 0.01 % leucine (w/v) in absence of thiamine. All subsequent cultures were grown in absence of thiamine to keep the nmt1 promoter controlling heterologous enzyme expression in an active state. Biomass production was scaled up by factor 10 to yield 100 mL or 1000 mL 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 $\times$ g for 5 min except for 1000 mL cultures where centrifugation was carried out at 5000 $\times$ g for 25 min. The biomass was washed three times with cold, deionized water and finally re-suspended in 0.1 M phosphate buffer (pH 8).

2.3 BIOTECHNOLOGICAL SYNTHESIS OF DRUG METABOLITES

2.3.1 Biotechnological Synthesis of HO-MPPP

2.3.1.1 Incubation of MPPP with CAD64

At first the substrate MPPP·HCl (63.4 mg, 250 µmol) was added to 1.0 L of CAD64 cell suspension (1.2×10^8 cells/mL) in 0.1 M phosphate buffer (pH 8) containing 2% glucose (w/v). The mixture was incubated in a 2 L Erlenmeyer flask at 30 ℃ over 48 h at a shaking velocity of 150 rpm. In-process control samples (100 µL) were drawn at time points 0 h, 9 h, 24 h, 36 h and 48 h and analyzed by HPLC-UV as described below. After 48 h, concentrated H_3PO_4 was added to a final pH of 3.5, the incubation mixture was then shaken for another 30 min at 150 rpm. Thereafter, it was centrifuged (8000 × g, 30 min) and the supernatant was used for further workup.

2.3.1.2 Extraction of MPPP and HO-MPPP

Four Bond Elut SCX HF cartridges (5 g, 20 mL) were put on a VacMaster10 vaccum manifold (Separtis; Grenzach-Wyhlen, Switzerland) and each conditioned with 20 mL of methanol and 20 mL of water. Thereafter, one quarter of the incubation supernatant was passed through each of the four cartridges under reduced pressure. The passed through supernatant from all four columns was combined and collected in 100 mL fractions. After the supernatant had completely been passed through, each cartridge was washed with two times 20 mL of 0.1 M HCl and the passed through washing solutions were collected and combined. Then each cartridge was washed twice with 20 mL of methanol and the passed through washing solvents were collected and combined. Finally, the retained compounds were eluted thrice with 40 mL of methanol-concentrated aqueous ammonia (96:4 v/v) per cartridge. The eluates of each elution step were collected and combined. In-process control samples of the 100 mL supernatant fractions and the washing solvents were directly analyzed by HPLC-UV as described below. A sample (100 µL) of each eluate fraction was evaporated to dryness (56 ℃) and reconstituted in 250 µL HPLC solvent prior to analysis.

2.3.1.3 Separation using semi-preparative HPLC

The combined eluates of the first and second elution step were evaporated to dryness (70 °C, reduced pressure). The residue was reconstituted in 8 mL of 50 mM ammonium formate buffer (pH 3.5)/acetonitrile (60:40 v/v). Aliquots (250 µL) were injected into the semi-preparative HPLC system consisting of a Hewlett Packard (HP) 1050 series injector (Agilent Technologies, AT; Waldbronn, Germany) equipped with a 1.0 mL loop, an AT 1200 series G1361A preparative pump, and an AT 1100 series diode array detector. The stationary phase was an AT Zorbax-300 SCX column (9.4 × 250 mm, 5 µm). The isocratic mobile phase consisted of 50 mM ammonium formate buffer (pH 3.5)/acetonitrile (80:20 v/v) delivered at a flow rate of 15 mL/min. The diode array detection range was from 190 nm to 900 nm with a target wavelength of 265 nm. After the first injection, all fractions with significant peaks at 265 nm were collected. The fractions containing HO-MPPP and MPPP were collected and combined with the corresponding fractions.

2.3.1.4 Isolation and Precipitation of HO-MPPP and MPPP

From the combined fractions of the semi-preparative HPLC containing either HO-MPPP or MPPP acetonitrile was removed by evaporation (70 °C, reduced pressure). NaOH (1 M) was added to the remaining aqueous part to a final pH of 12 and the solution was twice extracted with 200 mL of ethyl acetate using a 1000 mL separating funnel. Before and after each extraction step a sample of the aqueous phase was analyzed by HPLC-UV. After extraction, the organic phases were combined, dried over anhydrous Na_2SO_4, and subsequently evaporated to dryness (70 °C, reduced pressure). The residue was reconsti tuted in 5 mL of dry ethyl acetate and transferred to a 15 mL reaction tube. After adding one drop of 10 M HCl, the tube was sealed, vigorously shaken for 1 min, and left in the refrigerator over night. The next day, the tube was centrifuged (10,000 × g, 5 min) and the supernatant was removed. The precipitated product was washed twice with 1 mL of ethyl acetate and dried (75 °C, reduced pressure).

2.3.2 Biotechnological Synthesis of HO-MPHP

2.3.2.1 Incubation of MPHP with CAD64 and CAD66

At first the substrate MPHP·HNO_3 (80.5 mg, 250 µmol) was added to 1.0 L of CAD64 cell suspension (1.2×10^8 cells/mL) in 0.1 M phosphate buffer (pH 8) containing 2% glucose (w/v). The mixture was incubated in a 2 L Erlenmeyer flask at 30 °C over 66 h at a shaking velocity of 150 rpm. In-process control samples (0.5 mL) were drawn at time points 0 h, 17.5 h, 41 h, and 66 h and analyzed by HPLC-UV as described below. After 66 h, concentrated H_3PO_4 was added to a final pH of 3.5, the incubation mixture was then shaken for another 30 min at 150 rpm. Thereafter, it was centrifuged (8000 × g, 30 min) and the supernatant was used for further workup.

In a second approach the substrate MPHP·HNO_3 (80.5 mg, 250 µmol) was added to 1.0 L of CAD66 cell suspension (1.2×10^8 cells/mL) in 0.1 M phosphate buffer (pH 8) containing 2% glucose (w/v). The mixture was incubated in a 2 L Erlenmeyer flask at 30 °C over 48 h at a shaking velocity of 150 rpm. After 48 h, concentrated H_3PO_4 was added to a final pH of 3.5, the incubation mixture was then shaken for another 30 min at 150 rpm. Thereafter, it was centrifuged (8000 x g, 30 min) and the supernatant was used for further work up.

2.3.2.2 Extraction of MPHP and HO-MPHP

Four Bond Elut SCX HF cartridges (5 g, 20 mL) were put on a VacMaster10 vaccum manifold (Separtis; Grenzach-Wyhlen, Switzerland) and each conditioned with 20 mL of methanol and 20 mL of water. Thereafter, one quarter of the supernatant of the incubation of MPHP with CAD64 was passed through each of the four cartridges under reduced pressure. The passed through supernatant from all four columns was combined and collected in 100 mL fractions. After the supernatant had completely been passed through, each cartridge was washed with two times 20 mL of 0.1 M HCl and the passed through washing solutions were collected and combined. Thereafter, each cartridge was washed twice with 20 mL of methanol and the passed through washing solvents were collected and combined. Finally, the retained compounds were eluted thrice with 40 mL of methanol-concentrated aqueous ammonia (96:4 v/v) per cartridge. The eluates of each elution step were collected and combined. In-process control samples of the 100 mL supernatant

fractions and the washing solvents were directly analyzed by HPLC-UV as described below. A sample (100 µL) of each eluate fraction was evaporated to dryness (56 °C) and reconstituted in 250 µL HPLC solvent prior to analysis. The supernatant of the incubation of MPHP with CAD66 was worked up accordingly.

2.3.2.3 Separation using semi-preparative HPLC

The combined eluates of the first and second elution step were evaporated to dryness (70 °C, reduced pressure). The residue was reconstituted in 8 mL of 50 mM ammonium formate buffer (pH 3.5)/acetonitrile (60:40 v/v). Aliquots (250 µL) were injected into the semi-preparative HPLC system consisting of a Hewlett Packard (HP) 1050 series injector (Agilent Technologies, AT; Waldbronn, Germany) equipped with a 1.0 mL loop, an AT 1200 series G1361A preparative pump, and an AT 1100 series diode array detector. The stationary phase was an AT Zorbax-300 SCX column (9.4 × 250 mm, 5 µm). The isocratic mobile phase consisted of 50 mM ammonium formate buffer (pH 3.5)/acetonitrile (80:20 v/v) delivered at a flow rate of 15 mL/min. The diode array detection range was from 190 nm to 900 nm with a target wavelength of 263.5 nm. After the first injection, all fractions with significant peaks at 263.5 nm were collected. The fractions containing HO-MPHP were collected and combined.

2.3.2.4 Isolation and Precipitation of HO-MPHP

From the combined fractions of the semi-preparative HPLC acetonitrile was removed by evaporation (70 °C, reduced pressure). NaOH (1 M) was added to the remaining aqueous part to a final pH of 12 and the solution was twice extracted with 250 mL of ethyl acetate using a 1000 mL separating funnel. Before and after each extraction step a sample of the aqueous phase was analyzed by HPLC-UV. After extraction, the organic phases were combined, dried over anhydrous Na_2SO_4, and subsequently evaporated to dryness (70 °C, reduced pressure). The residue was reconstituted in 5 mL of dry ethyl acetate and transferred to a 15 mL reaction tube. After adding one drop of 37 M HCl, the content of the tube was evaporated to dryness and reconstituted in methanol. The liquid was then again partially evaporated and the tube filled up with ethyl acetate. Then the tube was sealed, vigorously shaken for 1 min, and left in the refrigerator over night. The next day, the

tube was centrifuged (10,000 × g, 5 min) and the supernatant was removed. The precipitated product was washed twice with 1 mL of ethyl acetate and dried (75 ℃, reduced pressure).

2.4 IDENTITY, STRUCTURE CONFIRMATION AND PURITY TESTS

2.4.1 Identity confirmation

Approximately 1 mg of product was dissolved in 1mL methanol. For identity confirmation by GC-MS, 100 µL of this solution were transferred to an autosampler vial and evaporated to dryness under a stream of nitrogen at 56 ℃. 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 using microwave irradiation at 440 W. The sample was then diluted with 900 µL of ethyl acetate. Aliquots (3 µL) were subsequently analyzed by GC-MS in the electron ionization (EI) and positive-ion chemical ionization (PICI) mode.

2.4.2 Structure confirmation

For structural confirmation by NMR, solutions of MPPP·HCl and MPHP·HNO_3 and their respective products were prepared in CD_3OD (HO-MPPP 5 mg/mL, HO-MPHP 10 mg/mL). ^{1}H-NMR (500 MHz) and ^{13}C-NMR spectra (125 MHz) were recorded on a Bruker DRX 500 (Bruker, Rheinstetten, Germany) at 300 K. The chemical shifts were given in δ values (ppm) relative to the solvent peaks at δ_H 3.30 and δ_C 49.00.

2.4.3 Purity tests

For purity check, 1 mg of product was dissolved in 1 mL of 50 mM ammonium formate buffer (pH 3.5)/acetonitrile (80:20 v/v) and 20 µL subsequently analyzed by HPLC-UV with conditions described below. Purity was determined as the percentage of the product peak area of the total peak area.

2.5 ANALYSIS BY HPLC-UV AND GC-MS

2.5.1 HPLC-UV analysis

2.5.1.1 Apparatus

A HP 1050 series HPLC system consisting of a quaternary pump, a degasser, an autosampler and a UV/vis detector operated at λ = 265 nm was used.

2.5.1.2 HPLC Conditions

All in-process control samples and the purity check sample were analyzed using an injection volume of 20 µL. The stationary phase was an AT Zorbax 300-SCX column (2.1 × 150 mm, 5 µm) and the isocratic mobile phase consisting of 50 mM ammonium formate buffer (pH 3.5)/acetonitrile (80:20 v/v) was delivered at a flow rate of 1.2 mL/min.

2.5.2 GC-MS analysis

2.5.2.1 Apparatus

Analysis for the trimethylsilylated sample extracts of the initial experiments and the trimethylsilylated final products, HO-MPPP and HO-MPHP respectively, was carried out using a HP 5890 Series II GC system combined with an HP 5989B MS Engine mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software.

2.5.2.2 GC-MS conditions

The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m x 0.2 mm I.D.), cross linked methylsilicone, 330 nm film thickness; injection port temperature, 280 ℃; carrier gas, helium; flow-rate 1 mL/min; column temperature, programmed from 100-310℃ at 30℃/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 energy, 70 eV; PICI: ionization energy, 230 eV; ion source temperature, 220 ℃; capillary direct interface heated at 260 ℃.

3 RESULTS

3.1 BIOTECHNOLOGICAL SYNTHESIS OF DRUG METABOLITES

3.1.1 Biotechnological Synthesis of HO·MPPP

3.1.1.1 Incubation of MPPP with CAD64

As shown in *Figure 1* in the in-process control samples there was an increase of the metabolite peaks between 0 h and 36 h, but no further increase was observed between 36 h and 48 h. The amount of parent compound decreased continuously until 48 h, but remained still in considerable abundance.

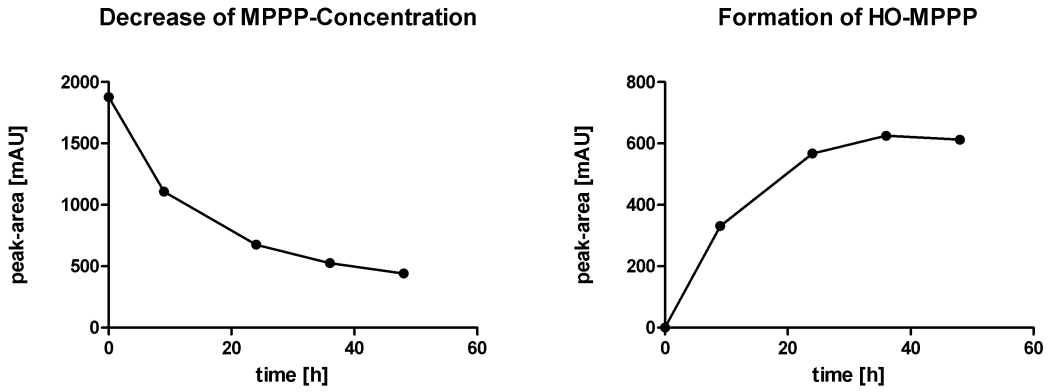


Figure 1 Peak areas of MPPP and HO-MPPP in HPLC-UV analysis (see 2.5.1) of in-process control samples taken during incubation of MPPP with CAD64.

3.1.1.2 Extraction of MPPP and HO-MPPP

Prior to the passage through the extraction cartridges supernatant samples were taken for HPLC-UV analysis. In comparison to HO-MPPP peaks in supernatant samples after the passage the extraction loss of HO-MPPP was negligible in the first 100 mL fraction and increased to approximately 20 % in the last fraction, resulting in total extraction loss over all fractions of about 10 %. Only very minor amounts of HO-MPPP were washed from the cartridges during washing steps. During elution the vast majority (about 90 %) of HO-MPPP could be eluted in the first elution fraction. The second fraction was found to only contain traces of HO-MPPP (<0.5 %).

3.1.1.3 Separation using semi-preparative HPLC

Baseline separation of HO-MPPP from co-extracted matrix compounds and parent drug MPPP was achieved within 15 min, the injection volume being 250 µL. This is illustrated in *Figure 2*.

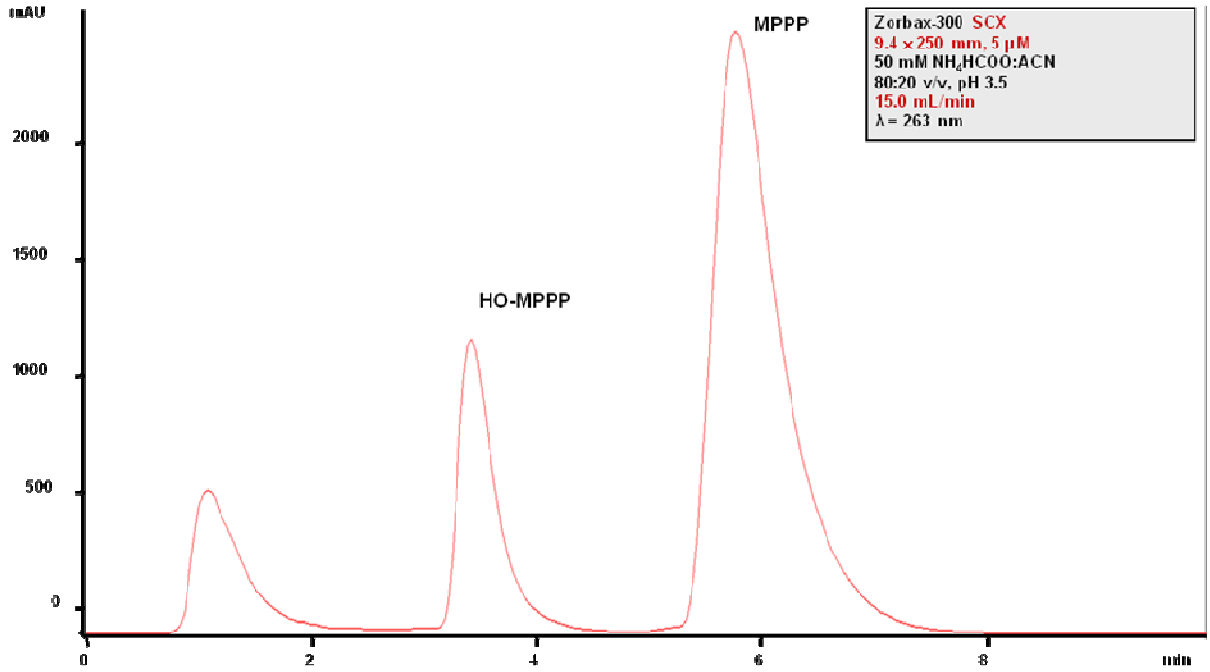


Figure 2 Chromatogram of semi-preparative HPLC cleanup of solid-phase extract reconstituted in 8 mL of HPLC solvent (injection volume, 250 µL). It can be seen that the HO-MPPP peak eluting at 4.0 min was baseline separated from matrix compounds and untransformed parent drug MPPP eluting at 6.0 min.

3.1.1.4 Isolation and Precipitation of HO·MPPP and MPPP

HPLC-UV analysis of the combined fractions from semi-preparative HPLC before and after each extraction step with ethyl acetate showed that a large percentage of HO-MPPP had already been extracted with the first extraction step. After the second extraction step detection of HO-MPPP was no longer detectable in the aqueous phase. Addition of concentrated HCl to the reconstituted organic extracts led to a milky suspension. Leaving the suspension in the refrigerator over night resulted in the formation of fine crystals. After centrifugation, removal of the supernatant, washing of the precipitate, and drying, the final product was obtained as an off-white crystalline powder. The yield was approximately 10 mg which corresponds to 37 µmol of HO-MPPP·HCl or 14.8 % of the theoretical maximum yield for the entire process. However, considering that only about 40 % of the initial MPPP were

biotransformed to HO-MPPP, this shows that the extraction and purification process gave a yield of 40 % of the metabolite present in the supernatant after incubation.

Also, in analogous preparation of the fractions from semi-preparative HPLC containing the parent drug MPPP, 4 mg of MPPP were obtained. This corresponds to 18.43 µmol and therefore about 7.5 % of the initial amount of added drug.

3.1.2 Biotechnological Synthesis of HO-MPHP

3.1.2.1 Incubation with of MPHP with CAD64 and CAD66

The parent compound MPHP and (trimethylsilylated) HO-MPHP could be identified in the supernatants of both strains. However, in the CAD64 sample, the peak corresponding to HO-MPHP was considerably more abundant and that of the parent drug in comparison to the CAD66 sample. Additionally, in the CAD66 incubation, minor amounts of a MPHP metabolite hydroxylated at the hexanone moiety and of two isomeric MPHP metabolites hydroxylated at the 4'-methyl and the hexanone moiety could be detected. Only the incubation supernatant of CAD 64 was worked up further.

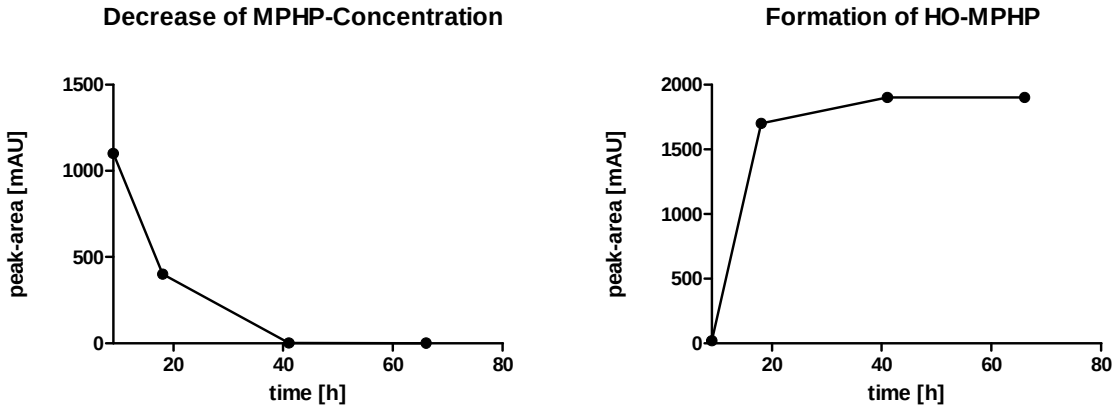


Figure 3 Peak areas of MPHP and HO-MPHP in HPLC-UV analysis (see 2.5.1) of in-process control samples taken during incubation of MPHP with CAD64.

As shown in *Figure* 3, the metabolite peaks in the in-process control samples of the incubation with CAD64 increased between 0 h and 41 h, but did not further increase between 41 h and 66 h. At the same time, the peak of the parent compound decreased to almost zero at 41 h and had entirely disappeared at 66 h. Apart from the peaks of the parent drug and the target metabolite, additional peaks were

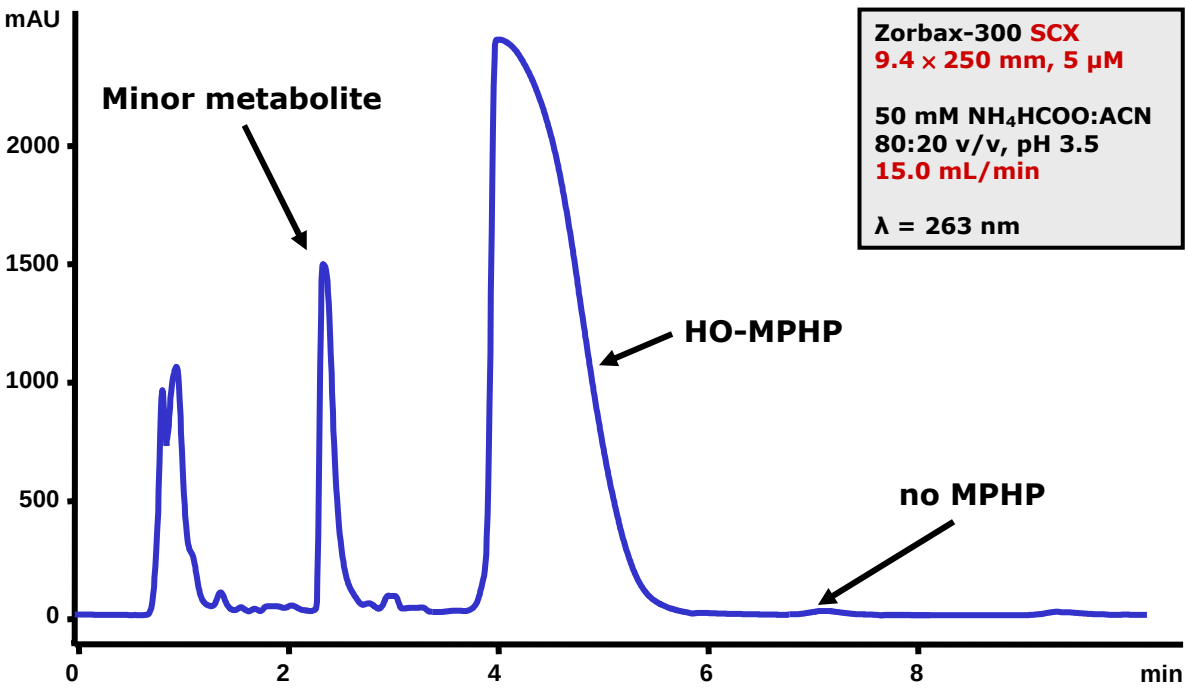
observed at approximately 0.6 min and at 1.2 min both of which continuously increased with incubation time. The peak eluting at 0.6 min was also observed in MPHP-free CAD64 incubations and can be attributed to fission yeast matrix. The peak eluting at 1.2 min was absent in MPHP-free incubations and therefore most likely attributable to an unknown MPHP metabolite.

3.1.2.2 Extraction of MPHP and HO-MPHP

Comparison of the HO-MPHP peaks in supernatant samples taken before and after passage through the extraction cartridges showed that the extraction loss of HO-MPHP was about 0.1 % in the first 100 mL fraction and increased to approximately 4 % in the last fraction. Total extraction loss over all fractions was approximately 1.3 %. During the washing steps only very minor amounts HO-MPHP were washed from the cartridges. During elution, the largest amount (about two thirds) of HO-MPHP was eluted in the first elution fraction. The second fraction still contained approximately one third of HO-MPHP, while only traces (<0.5 %) were found in the third elution fraction.

3.1.2.3 Separation using semi-preparative HPLC

As illustrated in *Figure 4*, baseline separation of HO-MPHP from co-extracted matrix compounds and the peak of the potential unknown metabolite was achieved within 10 min. The injection volume of 250 µL proved to be a good compromise between the total number of injections into semi-preparative HPLC and overload effects on the column.



. ***Figure 4.*** Chromatogram of semi-preparative HPLC cleanup of solid-phase extract reconstituted in 8 mL of HPLC solvent (injection volume 250 µL). It can be seen that the HO-MPHP peak eluting at 4.0 min was baseline separated from a presumed minor metabolite eluting at 2.3 min and from matrix compounds.

3.1.2.4 Isolation and precipitation of HO-MPHP

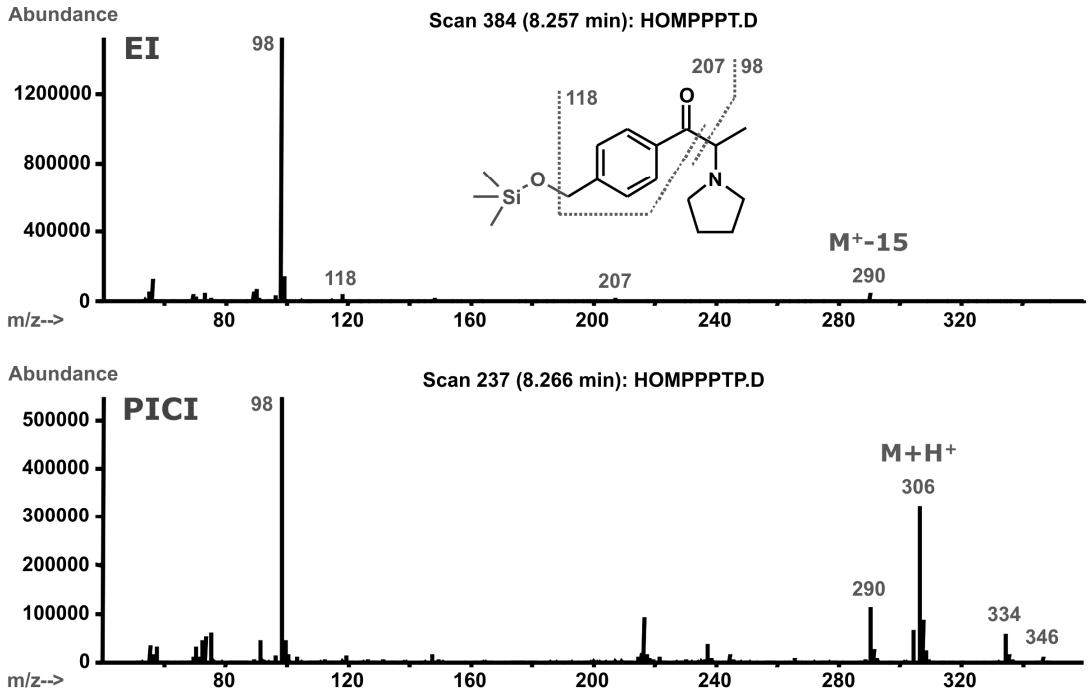
HPLC-UV analysis of the combined fractions from semi-preparative HPLC before and after each extraction step with ethyl acetate showed that a large percentage of HO-MPHP had already been extracted after the first extraction step. After the second extraction step, HO-MPHP was no longer detectable in the aqueous phase. Addition of concentrated HCl to the reconstituted organic extracts led to a milky suspension that cleared over night under formation of fine crystals. After centrifugation, removal of the supernatant, washing of the precipitate, and drying, the final product was obtained as an off-white crystalline powder. The yield was 42 mg which corresponds to 138 µmol of HO-MPHP·HCl or 55% of the theoretical maximum yield.

3.2 IDENTITY, STRUCTURE CONFIRMATION AND PURITY TESTS

3.2.1 Identity confirmation

3.2.1.1 Identity confirmation of HO-MPPP

The fragments of the EI mass spectrum proved to be in full agreement with chemical structure of trimethylsilylated HO-MPPP. The abundant signal at m/z 306 with the typical adduct ions at m/z 334 and m/z 346 in the PICI mass spectrum are in line with $[M+H]^+$, $[M+C_2H_5]^+$, and $[M+C_3H_5]^+$ of trimethylsilylated HO-MPPP confirming its molecular mass. The EI and PICI mass spectra of HO-MPPP are shown in *Figure 5*. No further relevant peaks were observed by GC-MS.



. ***Figure 5.*** EI (top) and PICI (bottom) mass spectra of trimethylsilylated HO-MPPP.

3.2.2.2 Identity confirmation of HO-MPHP

The fragments of the EI mass spectrum proved to be in full agreement with chemical structure of trimethylsilylated HO-MPHP. The abundant signal at m/z 348 with the and typical adduct ions at m/z 376 and m/z 388 in the PICI mass spectrum are in line with $[M+H]^+$, $[M+C_2H_5]^+$, and $[M+C_3H_5]^+$ of trimethylsilylated HO-MPHP confirming its molecular mass. The EI and PICI mass spectra of HO-MPPP are shown in *Figure 6*. No further relevant peaks were observed by GC-MS.

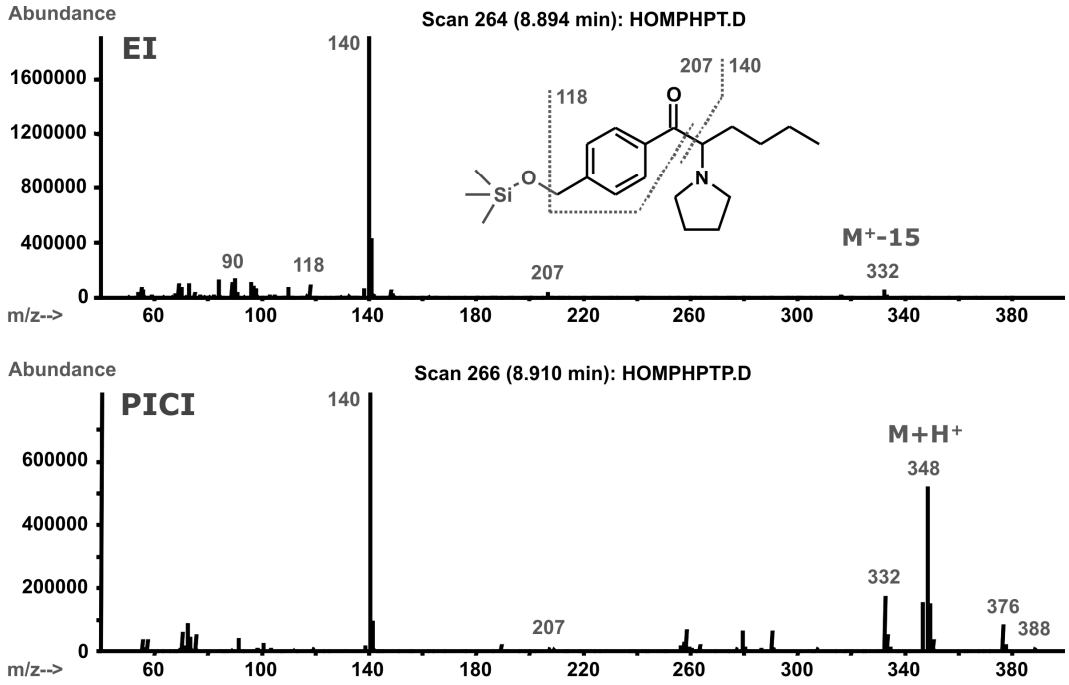


Figure 6. EI (top) and PICI (bottom) mass spectra of trimethylsilylated HO-MPHP.

3.2.2 Structure Confirmation

3.2.1.1 Structure confirmation of HO-MPPP

Figure 7 shows the ^{1}H-NMR spectra of the parent drug MPPP·HCl (top) and the isolated metabolite HO-MPPP·HCl (bottom) recorded during this study. Comparing these spectra, it can be seen that the three proton singlet at δ_H 2.45 ppm present in the spectrum of the parent drug has disappeared in the spectrum of the metabolite while a new singlet corresponding to two protons has appeared at δ_H 4.72 ppm. These findings are in exact agreement with the structure of HO-MPHP.

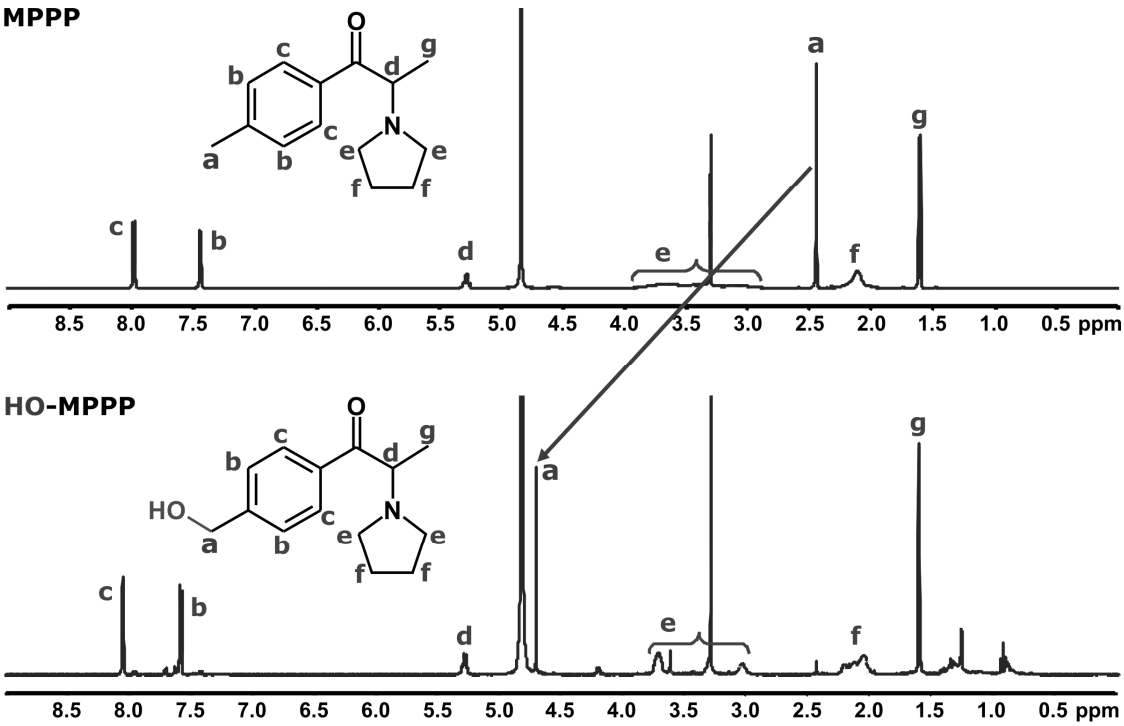


Figure 7. ^{1}H-NMR spectra of MPPP·HCl (top) and HO-MPHP·HCl (bottom). The labeling of the positions of the chemical structures corresponds to the labeling of proton signals in the respective panels. The shift of the three proton signal of the 4'-methyl group of MPPP at δ_H 2.45 ppm to a two proton signal at δ_H 4.72 ppm in the spectrum of HO-MPPP confirms the chemical structure of the latter.

3.2.2.2 Structure confirmation of HO-MPHP

Figure 8 shows the ^{1}H-NMR spectra of the parent drug MPHP·HNO_3 (top) and the isolated metabolite HO-MPHP·HCl (bottom) recorded during this study. Comparing these spectra, it can be seen that the three proton singlet at δ_H 2.45 ppm present in the spectrum of the parent drug has disappeared in the spectrum of the metabolite while a new singlet corresponding to two protons has appeared at δ_H 4.72 ppm. This is accompanied by a downfield shift of the respective ^{13}C signals from δ_C 21.78 (MPHP·HNO_3) to δ_C 64.31 (HO-MPHP·HCl) (data not shown). These findings are in exact agreement with the structure of HO-MPHP.

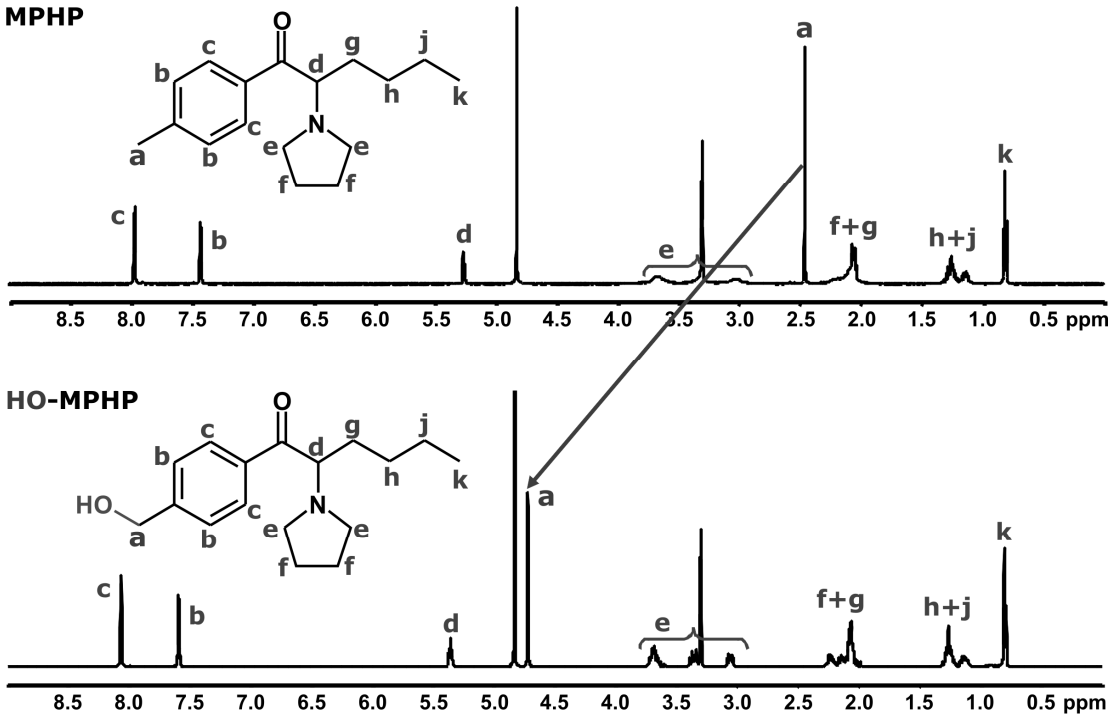


Figure 8. ^{1}H-NMR spectra of MPHP·HNO_3 (top) and HO-MPHP·HCl (bottom). The labeling of the positions of the chemical structures corresponds to the labeling of proton signals in the respective panels. The shift of the three proton signal of the 4'-methyl group of MPHP at δ_H 2.45 ppm to a two proton signal at δ_H 4.72 ppm in the spectrum of HO-MPHP confirms the chemical structure of the latter.

3.2.3 Purity tests

3.2.3.1 Purity test for HO-MPPP

With the cation-exchange Zorbax 300-SCX column the peak area of the product peak accounted for about 93 % of total peak area. The respective HPLC chromatogram is shown in *Figure 9*.

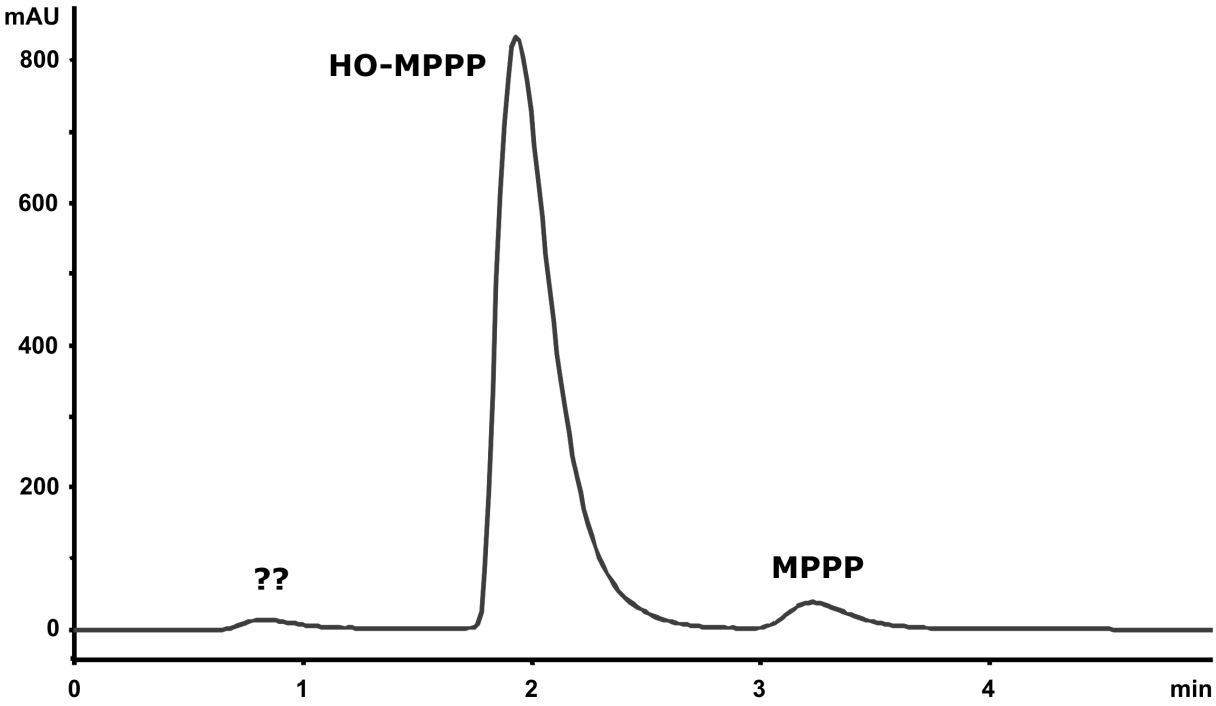


Figure 9. Chromatograms of a 1 mg/mL solution of the isolated product HO-MPPP·HCl on Zorbax 300-SCX column (2.1 × 150 mm, 5 µm), 50 mmol/L ammonium formate buffer (pH 3.5)/acetonitrile (80:20 v/v) delivered at 1.2 mL/min. The injection volume was 20 µL and UV detection was performed at 265 nm. The HO-MPPP peak accounted for about 93% of total peak area.

3.2.3.2 Purity test for HO·MPHP

With the cation-exchange Zorbax 300-SCX, the peak area of the product peak accounted for more than 99 % of total peak area. The respective HPLC chromatogram is shown in *Figure 10*.

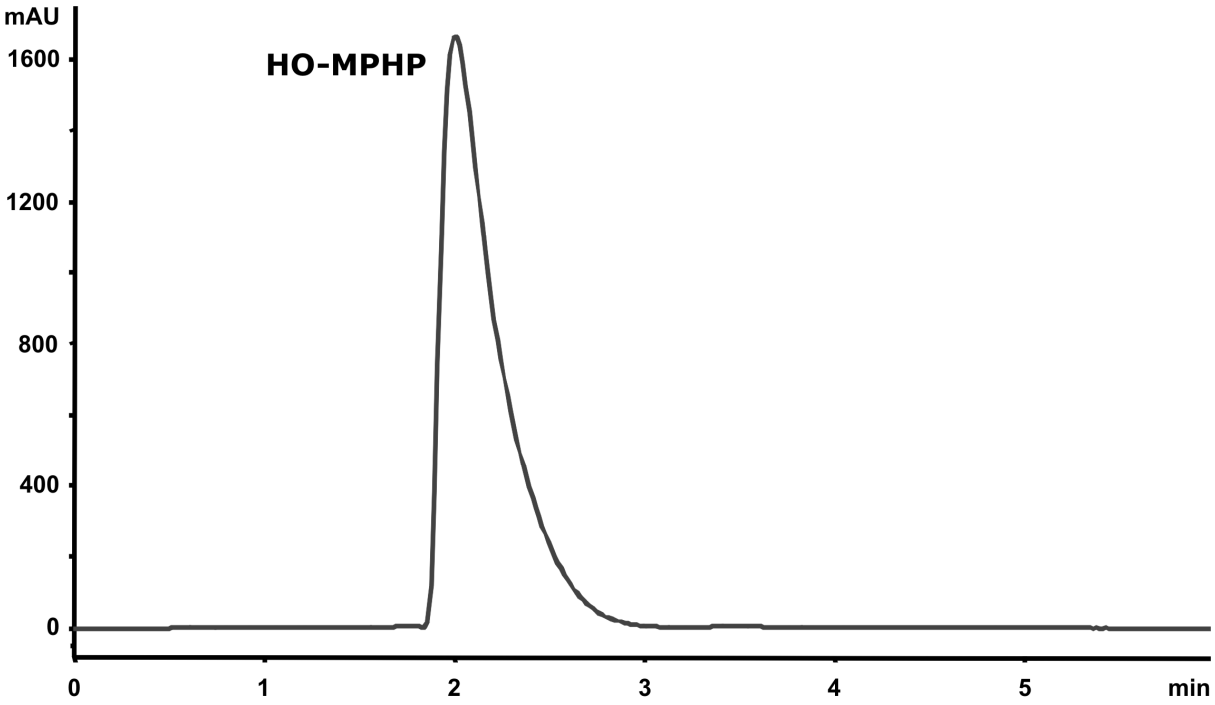


Figure 10. Chromatogram of a 1 mg/mL solution of the isolated product HO-MPHP·HCl on Zorbax 300-SCX column (2.1 × 150 mm, 5 µm), 50 mmol/L ammonium formate buffer (pH 3.5)/acetonitrile (80:20 v/v) delivered at 1.2 mL/min. The injection volume was 20 µL and UV detection was performed at 265 nm. The HO-MPHP peak accounted for more than 99% of total peak area.

4 DISCUSSION

The aim of the presented work was the biotechnological synthesis, isolation and purification of HO-MPPP and HO-MPHP, the metabolites of the new pyrrolidonophenone-type designer drugs MPPP and MPHP, respectively, and to therefore prove the feasibility of biotechnological synthesis of drug metabolites using microsomal human P450s heterologously expressed in the fission yeast *S. pombe*. The metabolites were needed as reference substance for studies on the quantitative involvement of human P450 isozymes in the main metabolic pathways of these drugs in humans. Moreover, they would be available for studies on the pharmacologic/toxcologic characterization of the metabolite as required by a recent guidance document of the US Food and Drug Administration for certain metabolites of therapeutic drugs [34,54].

The biotechnological synthesis of the drug metabolites as described here has several advantages over their classical chemical synthesis according to the protocol for the synthesis of the corresponding hydroxymethyl metabolite of the pyrrolidinophenone homologue pyrovalerone [30]. Enzyme catalyzed 4'-methyl-hydroxylation of MPPP and MPHP is only one step process while chemical synthesis involves eight different synthetic steps. In case of MPHP the total reaction time of both biotechnological and chemical synthesis is similar with 66 h and 62 h, respectively [34]. The total reaction time for biotechnological synthesis of HO-MPPP is even significantly lower with 48 h. Apart from that, chemical synthesis would have involved the time-consuming setup of more or less complicated chemical reaction apparatus as well as isolation, cleanup, and characterization of intermediate products. In contrast, biotechnological synthesis was performed in a simple Erlenmeyer flask and isolation and cleanup had to be performed only once. The synthetic protocol as described by *Michaelis et al.* would further have involved aggressive reaction conditions, such as refluxing of methanol with continual passage of HCl gas. Also the use of hazardous reagents and solvents, such as CuCN, $CHCl_3$, benzene, CaC_2 or Br_2 was necessary [30]. In contrast, the biotechnological synthesis ran smoothly at 30 °C and only comparatively non-hazardous chemicals and reagents were used for isolation and cleanup of the products.

To enable biotechnological synthesis the knowledge of main metabolic pathways of the respective drugs is essential. For MPPP and MPHP these metabolic pathways are already described in the literature [46,47,50,51]. In both cases 4'-methyl-

hydroxylation with subsequent oxidation to the respective carboxylic acid was the main metabolic pathway in the rat [46,51] and the formation of the 4'-methyl-hydroxy-metabolite can be assumed to be the initial reaction in humans. As reported by *Sauer et al.* [39] the detection of the 4'-carboxy metabolite in urine after ingestion of MPHP further implicates that oxidation is also the subsequent step in humans.

The biotechnological approach described here was based on the biotechnological synthesis of the 4'-hydromethyl metabolite of another pyrrolidinophenone-type drug, MPBP, using the fission yeast strain CAD58 heterologously expressing human P450 2D6 [33]. In the present work the fission yeast strains CAD64 and CAD66 heterologously co-expressing P450 2D6, P450 2C19, respectively, and its redox partner hCPR were used [34]. P450 2D6 and 2C19 are among the most relevant in the metabolism of many therapeutic and illicit drugs in humans [16,26,36]. Previous studies on the metabolism of MPPP and MPHP show that both isozymes account for the vast majority of the formation of the respective 4'-hydroxymethyl metabolite, in case of MPPP even the only two CYP P450 isozymes found to be involved [46,51]. In the metabolism of MPPP, the main catalyzing isozyme according to these studies seems to be P450 2C19. The fact that in the present study a fission yeast strain only expressing P450 2D6 was used could be one reason why the parent drug MPPP was not metabolised completely during incubation. In the biotechnological synthesis of HO·MPHP CAD64 was preferred over CAD66, because it had shown higher conversion of MPHP to HO·MPHP and less formation of side products than the latter [34].

The metabolic pathways catalyzed by the isozymes and P450 2D6 and 2C19 themselves are also of particular interest in pharmacologic and toxicological research for their polymorphically expression [16]. In so-called poor metabolizer subjects of these isozymes the hepatic clearance of drugs catalyzed by them can be significantly lower. For P450 2D6 and 2C19 poor metabolizer subjects account for about 7% and 3% of the Caucasian population, respectively [2,43]. Thus users of these drugs with poor metabolizer genotypes might stand at higher risk of developing adverse or toxic effects. However, further assessment of the clinical relevance of these genetic polymorphisms regarding the toxicokinetics of MPPP and MPHP would require clinical trials. This, however, seems at least problematic from an ethical point of view.

Co-expression of the accessory protein hCPR generally enhances the activity of heterologously expressed human P450 enzymes [5,8,17,53]. Even though the

presence of hCPR was obviously not critical in biotechnological synthesis of HO-MPBP, this can not necessarily be expected for other substrates.

The SPE procedure with a strong cation exchange extraction sorbent effectively retained the basic metabolite HO·MPHP as indicated by the very minor amounts in the supernatant after passing through the extraction cartridges. As described, in case of HO-MPPP there was a much higher loss of product. HO-MPPP and HO-MPHP were also effectively retained during the washing steps that however removed the vast majority of matrix compounds. In the final step, the retained compounds could be eluted with comparatively small volumes of elution solvent. The overall solvent consumption was considerably lower than in the back extraction described in the previous study of the biotechnological synthesis of HO-MPBP [33].

Because of formation of an unknown side product in the case of MPHP and incomplete removal of matrix compounds in both cases, the raw extract had to be cleaned up by semi-preparative HPLC. In the case of HO-MPPP this could be achieved by 32 injections with a run time of 15 min, for HO-MPHP even with a run time of 10 min, so that product cleanup could be completed in one work day in both cases.

In a first attempt it was tried to isolate HO-MPPP from the collected HPLC fractions by SPE analogous to the method of solid-phase extraction from the fission yeast culture supernatant. But it proved too much loss of product due to competition of the metabolites with ammonium ions from the mobile phase buffer at the cation exchange sorbent. So for the further isolation of the metabolites HO-MPPP and HO-MPHP from the collected HPLC fractions liquid-liquid extraction was preferably performed.

Precipitation of the final product as hydrochloric salt was performed in the same way as previously described for HO·MPBP [33]. The final product yield of 14.8 % of HO-MPPP and 55 % of HO·MPHP seems to stand in contrast to the results of the in-process control samples indicating effective solid-phase extraction, elution and liquid-liquid extraction. However, in both cases it must be assumed that a considerable amount of product might have been separated with the fission yeast cells. These were not included in the workup to avoid interference from cell matrix compounds. A certain amount was probably also lost during HPLC cleanup and, most importantly, product precipitation. In addition, in case of MPPP the parent drug had not been completely metabolized so that after precipitation about 7.5 % of the amount of MPPP initially added to the fission yeast culture were still obtained. In any case, the

product yield of 55% in case of HO-MPHP is almost twice as high as the 30% that could have been expected with chemical synthesis according to the protocol described in the referred study [30]. Taking into account that only about 40 % of the initial MPPP were biotransformed to HO-MPPP, it can be assumed that the product yield really after extraction and purification process is 40 % of the theoretical maximum yield, which is still slightly more than the expected yield with chemical synthesis. Moreover, the 10 mg of HO-MPPP·HCl and the 42 mg of HO-MPHP·HCl obtained in the present study were sufficient for product characterization by GC-MS, NMR and HPLC-UV and for use as reference substance in further studies on MPPP and MPHP 4'-methyl hydroxylation in human organism.

Concerning product identity, the fragmentation pattern and molecular mass of the trimethylsilylated products observed in GC-MS analysis and the signal shifts as compared to the parent drug in NMR analysis gave unambiguous proof that the isolated metabolites were indeed HO-MPPP and HO-MPHP. Concerning product purity, the fact that the peak area of HO-MPPP accounted for 93% of total peak area and that of HO-MPHP even for more than 99% of total peak area in HPLC analysis indicates the very high purity of the isolated products. This was further confirmed by the GC-MS data and the NMR data, which did not give any indication of relevant impurities.

5 CONCLUSION

In conclusion, the presented data show that the biotechnological synthesis of HO-MPPP and HO-MPHP using fission yeast strains CAD64 and CAD66 co-expressing human P450 2D6 and 2C19, respectively, and hCPR yielded the product in an acceptable amount and high purity. In comparison to classical chemical synthesis, the biotechnological approach was less time-consuming, less complicated and safer considering the mild reaction conditions and avoidance of hazardous chemicals and solvents. Biotechnological synthesis employing one of these new fission yeast strains may help overcome complicated and time-consuming chemical methods in the synthesis of drug metabolites.

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7 ABBREVIATIONS

PPP	α-pyrrolidinopropiophenone, 1-phenyl-2-pyrrolidin-1-ylpropanone (IUPAC)
PVP	α-pyrrolidinovalerophenone, 1-phenyl-2-pyrrolidin-1-ylpentanone (IUPAC)
MPPP	4'-methyl-α-pyrrolidinopropiophenone, 1-(4-methylphenyl)-2-pyrrolidin-1-ylpropan-1-one (IUPAC)
HO-MPPP	4’-hydroxymethyl-α-pyrrolidinopropiophenone, 1-(4-hydroxymethylphenyl)-2-pyrrolidin-1-ylpropan-1-one (IUPAC)
MOPPP	4'-methoxy-α-pyrrolidinopropiophenone, 1-(4-methoxyphenyl)-2-pyrrolidin-1-ylpropan-1-one (IUPAC)
MDPPP	3',4'-methylenedioxy-α-pyrrolidinopropiophenone, 1-(1,3-benzodioxol-5-yl)-2-pyrrolidin-1-ylpropan-1-one (IUPAC)
MDPV	3’,4’-methylenedioxypyrovalerone 1-(3,4-methylenedioxyphenyl)-2-pyrrolidinylpentan-1-one (IUPAC)
MPBP	4'-methyl-α-pyrrolidinobutyrophenone, 1-(4-methylphenyl)-2-pyrrolidin-1-ylbutan-1-one (IUPAC)
HO-MPBP	4’-methyl-α-pyrrolidinobutyrophenone, 1-(-4-hydroxy-methylphenyl)-2-pyrrolidin-1-ylbutan-1-one (IUPAC)
MPHP	4'-methyl-α-pyrrolidinohexanophenone, 1-(4-methylphenyl)-2-pyrrolidin-1-ylhexan-1-one (IUPAC)
HO-MPHP	4’-hydroxymethyl-α-pyrrolidinohexanophenone, 1-(4-hydroxymethylphenyl)-2-pyrrolidin-1-ylhexan-1-one (IUPAC)
hCPR	Human Cytochrome P450 reductase
GC-MS	Gas Chromatographic-Mass Spectrometry
MSTFA	*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide
EMM	Edinburgh minimal medium;

HP	Hewlett Packard
AT	Agilent Technologies
LLE	Liquid-liquid Extraction
SPE	Solid-phase Extraction

8 PUBLIKATIONEN

Peters FT, Dragan CA, Kauffels A, Schwaninger AE, Zapp J, Bureik M, Maurer HH (2009) Biotechnological synthesis of the designer drug metabolite 4'-hydroxymethyl-α-pyrrolidinohexanophenone in fission yeast heterologously expressing human cytochrome P450 2D6 – a versatile alternative to multistep chemical synthesis. J Anal Toxicol. 33:190-197

9 DANKSAGUNG

Die vorliegende Arbeit entstand unter Anleitung von Herrn Privatdozent Dr. Frank T. Peters in der Abteilung für Experimentelle und Klinische Toxikologie der Universität des Saarlandes in Homburg/Saar (Leiter Prof. Dr. Dr. h.c. Hans H. Maurer).

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