Enantioselective bioreduction of propentofylline using yeast in water and organic solvents

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Summary

Propentofylline (PPT) is a drug used in the treatment of both vascular dementia and Alzheimer type dementia. Hydroxy-metabolites of propentofylline (OHPPT) also demonstrate the same biological activity as the parent compound. As steroisomers of HOPPT are not commercially available, we had to produce them for pharmacological and pharmacokinetic studies. The aim of this study was to find the strains of *Saccharomyces cerevisiae* yielding enantiomerically pure (R)- or (S)-1-(5-hydroxyhexyl)-3-methyl-7-propylxanthin (OHPPT) from PPT. In this paper, we present the results of stereoselective reduction of PPT into OHPPT when catalysed by whole cells of baker's and a few strains of wine yeast in water and organic solvents.

Key words:
propentofylline, metabolites of propentofylline, *Saccharomyces cerevisiae*, enantiomeric excess.

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1. Introduction

Propentofylline (PPT, 3-methyl-1-(5-oxohexyl)-7-propyl-xanthine) has been reported to be beneficial in the treatment of both vascular dementia and Alzheimer type dementia (1-4). PPT increases the solubility of lipids by substituting a methyl group for a propyl one in position 7 of the purine backbone of pentoxifylline. The pharmacological effects of PPT may be observed in

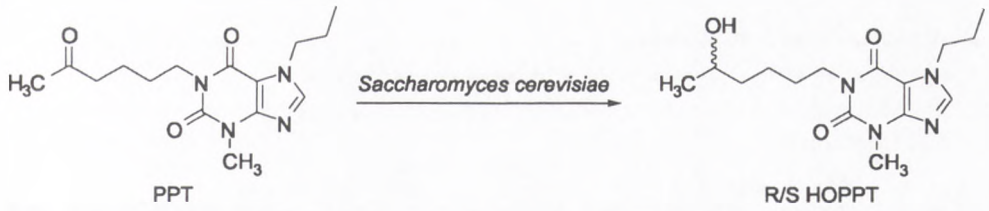

Fig. Bioreduction pathway of propentofylline by yeast.

stimulation of the nerve growth factor, increased cerebral blood flow and inhibition of adenosine uptake. PPT also enhances extracellular adenosine concentrations and decreases extracellular levels of glutamate *in vivo* during ischemia (5,6).

To date, despite the known pharmacological effects, few clinical pharmacokinetic or metabolism studies of PPT have been reported in the literature. The short half-life of PPT at the terminal elimination phase and poor bioavailability, after oral administration to rabbits, suggest that this drug undergoes extensive first-pass metabolism in liver. PPT is metabolised *in vitro* to the racemic compound – (±)-1-(5-hydroxyhexyl)-3-methyl-7-propyl-xanthine (HOPPT) (7-10).

Studies have shown that the racemic mixture and the steroisomers of HOPPT demonstrate biological activity. Hydroxy-metabolites of PPT inhibited [^{3}H]nitrobenzylthioinosyne binding in rat brains with a similar affinity to propentofylline, and also inhibited [^{3}H]adenosine uptake by es transport as effectively as propentofylline. Since inhibition of adenosine transport appears to be important for the neuroprotective effects of PPT, the hydroxy-metabolites may also provide neuroprotection. As steroisomers of HOPPT are not commercially available, we had to produce them for pharmacological and pharmacokinetic studies.

In this paper, we present the results of stereoselective biotransformation of PPT to HOPPT when catalysed by whole cells of baker's and a few strains of wine yeast in both water and organic solvents. Alcohol dehydrogenases (YADH's) in yeast (*Saccharomyces cerevisiae*) play an important role in the reduction of carbonyl functions in aldehydes and ketones (11). YADH has very narrow substrate specificity and generally accepts only aldehydes and methyl ketones. It is therefore of only limited use in the preparation of the chiral secondary alcohols.

Microbiological reduction with yeast was carried out under non–fermenting conditions. The stereoselectivity of this biotransformation process was determined using the HPLC technique with a chiral column. It was established that different yeast strains favoured the biotransformation of PPT into different enantiomers.

2. Materials and methods

2.1. Chemicals

Propentofylline was obtained from Intervet GmbH, Germany; n-hexane and 2-propanol of HPLC grade were purchased from Merck KgaA Darmstadt, Germany. Other reagents were all of analytical grade.

2.2. Microorganisms

Five strains of *Saccharomyces cerevisiae*: KKP13, KKP35, KKP82, KKP295, KKPU were obtained from the Collection of Productive Microorganisms, Department of Technical Microbiology and Biochemistry in Warsaw, Poland; another two strains: L'hirondelle and Wołczyn were taken from Lesaffre Biocorporation in Warsaw, Poland; and Krakowskie was obtained from Baker's Company in Cracow, Poland.

2.3. Chemical synthesis

1-(5-R,S-hydroxyhexyl)-3-methyl-7-propylxanthine (HOPPT) – racemic standard. This compound was synthesised from PPT using sodium borohydride reduction. Briefly, 140 mg (0.5 mmol) of PPT was dissolved in 10 mL of methanol and 160 mg (2.5 mmol) of sodium borohydride was added. The mixture was stirred overnight at room temperature. The progress of the reaction was followed using thin layer chromatography (benzene:acetone 1:1). After solvent evaporation, the reaction product was extracted with methylene chloride. The organic layer was washed three times with saturated sodium chloride solution and concentrated under reduced pressure. The residue was recrystallized from ethyl ether. M.p. 77-79°C; 90% yield; HPLC: t_r = 14.50; 17.25 min; IR (KBr) [cm^{-1}]: 3372 (OH), 1699 (CO), 1645 (CO); ^1H NMR 300 MHz [$DMSOd_6$]: δ [ppm]: 0.83 (t, J=7.2 Hz, 3H, CH_3-CH), 1.03 (d, J=6.3 Hz, 3H, CH-CH_3), 1.30 (m, 4H, CH_2-CH_2-CH_2-CH_2), 1.50 (m, 2H, CH_2-CH_2-CH_2), 1.78 (q, J=7.4, 4H, CH-CH_2- CH_2), 3.43 (s, 3H, N-CH_3), 3.56 (q, J=5.5 Hz, 1H, CH), 3.85 (t, J=7.2 Hz, 2H, N-CH_2-CH_2), 4.21 (t, J=7.1 Hz, 2H, N-CH_2-CH_2), 4.33 (d, J=7.2 Hz, 1H, OH), 8.09 (s, 1H, Im-H).

2.4. Microbiological methods

2.4.1. Biotransformation of PPT in an aqueous medium

In a typical experiment, 2 g of wine yeast (KKP) or 5 g of baker's yeast were suspended in 150 mL of phosphate buffer. After stirring for 30 min at 30°C, 5 g of glucose and 235 mg (1 mmol) of PPT were added. After stirring for 7 days at 30°C, the yeast was filtered off. The filtrate was extracted three times with 100 mL of methylene chloride and dried over Na_2SO_4. The residues obtained from the evaporation of the solvent were either dissolved in 2 mL 2-propanol for HPLC analysis or purified using silica gel chromatography.

2.4.2. Biotransformation of PPT in organic media

Dry baker's or wine yeast (2 g) was suspended in 25 mL of the organic solvent and 2 mL of tridestilled water was added. After the addition of 58 mg (0.25 mmol) of PPT the suspension was stirred for 3 days at 27°C. The yeast was then filtered off, the solution concentrated *in vacuo*, and the residue was dissolved in 2.0 mL of 2-propanol for analytical purposes.

2.5. Methods of analysis

Polarimetry: Measurement of the optical rotation of the products of the stereoselective reduction PTX was carried out on a Digital Polarimeter – DIP 2000 (Jasco Inc., Japan). The optical rotations R- and S-1-(5-hydroxyhexyl)-3-methyl--7-propylxanthine were in accordance with the literature values (11).

Spectroscopy: ^1H NMR spectra for the racemic standard and each enantiomer were recorded using a Varian VM 300 Hz instrument using tetramethylsilane (TMS) as an internal standard. The IR spectra were recorded in KBr pellets on a Jasco Spectrometer FT/IR-410.

High performance liquid chromatography: The identification of the starting materials and reduction products as well as the yields of transformation and enantiomeric excess were determined by HPLC analysis on a Daicel ChiralPack AD Column. The high-performance liquid chromatograph (Dionex Corporation, USA) consisted of an isocratic solvent delivery system (Dionex HPLC Pump Series P580), an inlet equipped with a 20 μL loop and a variable wavelength UV detector (model Dionex UV/VIS detector UVD 170S/340S), set at 275 nm. The analytical chiral column was 250 × 4.6 mm i.d. Daicel ChiralPack AD (Chemical Industries, France). The temperature was set at 25°C. The mobile phase was n-hexane:2-propanol 780:220 per

1L phase, *vacuum*-degassed before use, with a flow rate of 1mL min^{-1}. The analytes were disolved in 2-propanol (at a concentration of 1mg/mL).

Chromatographic characteristics: Chromatographic data are given in Table 1.

Table 1

Chromatographic data

k_1	k_2	α	R_S	$-\Delta(\Delta G°)$ (J· mol^{-1})
3.02	4.02	1.33	2.25	630.53

The chromatographic conditions: n-hexane/2-propanol 780/220 (v/v);

flow rate 1.0 mL/min (isocratic); column temperature 25°C; UV detection at 275 nm; injection 20 µl.

The separation factor (α) was expressed as $\alpha = k_2 / k_1$, where k_2, k_1 are the retention factors for the first and second eluting enantiomer.

The retention factors k_1 and k_2 were calculated as follows:

$k_1 = (t_{R1} - t_0) / t_0$ and $k_2 = (t_{R2} - t_0) / t_0$, where t_0, t_{R1} and t_{R2} are the dead elution times of enantiomers.

The resolution (R_s) of the first and second eluting enantiomers was calculated by the ratio of the difference between the elution times t_{R1} and t_{R2} to the arithmetic mean of the two peaks' widhts w_1 and w_2.

$R_s = 2(t_{R2} - t_{R1}) / (w_1 + w_2)$

The difference in the free energy ($-\Delta(\Delta G°)$) was calculated from the separation factor according to the following equation: $-\Delta(\Delta G°) = RT \ln\alpha$

3. Results and discussion

1-(5-Oxohexyl)-3-methyl-7-propylxanthine (PPT) was reduced with $NaBH_4$ to R,S-1-(5-hydroxyhexyl)-3-methyl-7-propylxanthine (HOPPT), which was used as the reference standard in further analytical studies.

The aim of this study was to find the strains of *Saccharomyces cerevisiae* yielding enantiomerically pure (R)- or (S)-1-(5-hydroxyhexyl)-3-methyl-7-propylxanthine from PPT. Microbiological reduction using yeast was carried out under non-fermenting conditions. In Table 2, a summary of the results obtained using aqueous and organic media is given. PPT was reduced using baker's yeast (*Saccharomyces cerevisiae*: strains – L'hirondelle, Wołczyn and Krakowskie), in an aqueous medium according to Prelog's rule (12) to give an (S)-alcohol with a good optical purity only in the case of the L'hirondelle strain, with an ee of 78% for the (S) enantiomer. Bioreductions using baker's yeast were also performed in organic solvents such as ethyl acetate and n-hexane. Table 2 shows that in several cases such reductions can be carried out quite successfully. However, in organic media yields of the reduction of PPT to HOPPT were quite low (from 1 to 23%). In ethyl acetate, all the baker's strains preferred to form an (S) isomer, whereas in the n-hexane L'hirondelle strain an (R) isomer was preferred. Drastic solvent dependent ee variations were found for the re-

duction of PPT using the L'hirondelle strain: 78% ee in favour of the (S) enantiomer in water and 100% ee favouring the (R) enantiomer in n-hexane.

Table 2

Bioconversion of PPT to OHPPT in water and in organic solvents

Strains	Yields in water		Yields in AcOEt		Yields in n-hexane	
	[%]	ee[%]	[%]	ee[%]	[%]	ee[%]
KKP 13	32	9 (R)	nd		nd	
KKP 35	48	50 (R)	nd		nd	
KKP 82	94	67 (R)	nd		nd	
KKP 295	75	31 (S)	nd		nd	
KKPU	59	14 (R)	nd		nd	
L'hirondelle	67	78 (S)	10	100 (S)	1	100 (R)
Wołczyn	37	42 (S)	23	100 (S)	2	100 (S)
Krakowskie	41	44 (S)	1	100 (S)	1	100 (S)

nd: not determined

PPT was also bioreduced in the presence of wine yeast in an aqueous medium contrary to Prelog's rule to give the (R)-alcohol, as is the case with reduction using *LKADH* (alcohol dehydrogenase from *Lactobacillus kefiri*) (13,14). The biotransformation of PPT in water using wine yeast gave (R) HOPPT with variable enantiomeric excesses (ees from 14 for KKPU to 67% for KKP82), and yields ranging from 32 to 94%. Only in the case of biotransformation with KKP295, an (S) isomer (ee=31%) was obtained. The KKP82 strain was found to most favour the production of (R) HOPPT.

4. Conclusions

The above results demonstrate that the reduction of PPT in ethyl acetate with the yeast strain Wołczyn offers a new highly stereoselective method of (S)-1-(5-hydroxyhexyl)-3-methyl-7-propylxanthine preparation. Other reduction conditions employed were less effective.

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