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ENANTIOSELECTIVE BIOTRANSFORMATION OF PENTOXIFYLLINE INTO LISOFYLLINE USING WINE YEAST BIOCATALYSIS

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Abstract: Lisofylline (1-(5-R-hydroxyhexyl)-3,5-dimethylxanthine (**LSF**)) is a new methylxanthine, a stereospecific isomer which is a metabolite of pentoxifylline (1-(5-oxohexyl)-3,5-dimethylxanthine (**PTX**)). Alcohol dehydrogenases (E.C. 1.1.X.Y.) are enzymes that catalyze the oxidation and reduction of hydroxyl and carbonyl compounds. They may be employed either as crude or purified enzymes or as components of whole cells. The aim of this study was to explore the stereoselective bioreduction of **PTX** in the presence of whole cell baker's and wine yeasts, which function as biocatalysts in the production of **LSF**. The experiments were conducted in water and a number of organic solvents (toluene, hexane, ethyl acetate), and we obtained **LSF** with different yields and ee values. Our research demonstrated that the highest activity is shown when the KKPU strain is used in an aqueous medium. The biotransformation of **PTX** into **LSF** in this case was characterized by high yield and enantioselectivity: 95% and ee = 98%, respectively.

Keywords: pentoxifylline, lisofylline, Saccharomyces cerevisiae, enantiomers, enatiomeric exscess

Lisofylline, 1-(5-R-hydroxyhexyl)-3,5-dimethylxanthine (**LSF**) (Figure 1) is a novel, modified methylxanthine with anti-inflammatory properties. It was originally developed to reduce cellular damage due to ischemic reperfusion, hypoxia or autoimmune diseases (1, 2). Only the R stereoisomer is biologically active. **LSF** is several hundred-fold more effective than its parent compound, pentoxifylline (**PTX**), at inhibiting responses to the release of inflammatory cytokines (2). **LSF** inhibits stress-activated lipid metabolism and suppresses the production of inflammatory cytokines, such as interleukin 12 (IL-12) (3), which exert these effects through a common lipid intracellular signalling pathway. It also reduces toxicity and improves patient response to cancer chemotherapy and radiation therapy (4, 5). **LSF** can decrease the dysfunction caused by IL-1β in pancreatic islets, which indicates possible therapeutic value in the prevention of autoimmune disorders, including type I diabetes, in autoimmune recurrence following islet transplantation, and in the preservation of β cell functional mass during isolation (6-8).

LSF can be synthesized in several different ways, using chemical multiple-step methods (9, 10) or a microbial procedure with *Rhodotula rubra* (11). As **LSF** is not commercially available in our country we had to produce it for pharmacokinetic studies. A new, simple method was developed based on the enantioselective reduction of 1-(5-oxohexyl)-3,7-dimethylxanthine (**PTX**) using baker's and wine yeasts as biocatalysts (Figure 2). Among numerous enzymes in yeast *(Saccharomyces cerevisiae)* alcohol dehydrogenases (YADH) play an important role in the oxidation and reduction of hydroxyl or carbonyl functions in aldehydes, ketones, keto esters and keto acids (12-17). YADH has very narrow substrate specificity and generally accepts only aldehydes and methyl ketones (18, 19). Thus, YADH is of limited use in the preparation of chiral secondary alcohols (which include **LSF**). YADHs are ubiquitous enzymes involved in many physiological processes: they contain four Zn(II) atoms near the active site (20). Alcohol dehydrogenases may be employed as purified crude enzymes or as components of whole cells. The fundamental difference between the use of isolated enzymes and whole cells is that in the former case the cofactors necessary for the regeneration of the NAD(P)/NAD(P)H must be provided, whilst in the latter the cells contain the

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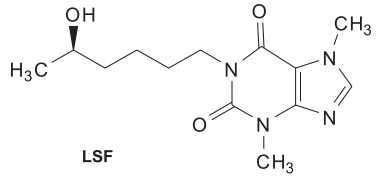

Figure1. Chemical structure of lisofylline.

necessary enzymes to achieve cofactor recycling. The disadvantage of using whole cells is that they frequently contain more than one oxidoreductase and the activity displayed depends on a number of factors including the structure of the substrate and the metabolic state of the cell. Nevertheless, procedures using whole microorganisms, for example *Saccharomyces cerevisiae,* are simple and inexpensive. In addition, their use does not require sterile fermentors and therefore they can be handled using standard laboratory equipment.

This study presents the results of the stereoselective reduction of **PTX** into **LSF** when catalyzed by whole cells of baker's yeast and a few strains of wine yeast, in both water and organic solvents. The enantiopure **LSF** was examined using the HPLC method with a chiral stationary phase.

EXPERIMENTAL

Materials

Pentoxifylline was obtained from Polpharma S.A., Poland; n-hexane and 2-propanol of HPLC grade were purchased from Merck, Darmstadt, Germany. The other reagents were all of analytical grade. 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 Asmussen, were obtained from Lesaffre Biocorporation, France and Oetker Nahrungsmittel K. G., Germany, respectively.

Methods of analysis

High performance liquid chromatography

The identification of starting materials and reduction products, each yields of transformation and enantiomeric excess, were determined by HPLC analysis on a Daicel Chiralpak AD column. The high-performance liquid chromatograph (Dionex Corporation, USA) consisted of an isocratic solvent delivery system (Dionex HPLC Pump Series P580), an injector 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 mm x 0.46 cm i.d. Daicel Chiralpak 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 and the flow rate was 1 mL min^{-1}. The analytes were dissolved in 2-propanol (at a concentration of 1mg/mL)**.**

Chromatographic characteristics

All chromatographic data are presented in Table 2.

The separation factor (α) was expressed as

$$\alpha = k_2 / k_1 \quad (1)$$

where k_1, k_2, are the retention factors for the first and second eluting enantiomer, respectively. The retention factors k_1 and k_2 were calculated as follows:

$$k_1 = (t_{R1} - t_0) / t_0 \text{ and } k_2 = (t_{R2} - t_0) / t_0 \quad (2)$$

where t_0, t_{R1} and t_{R2} are the dead elution time of enantiomers.

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

$$Rs = 2(t_{R2} - t_{R1)} / (w_1 + w_2) \quad (3)$$

The difference in the free energy ($-\Delta(\Delta G^o)$) was calculated from the separation factor according to the following equation:

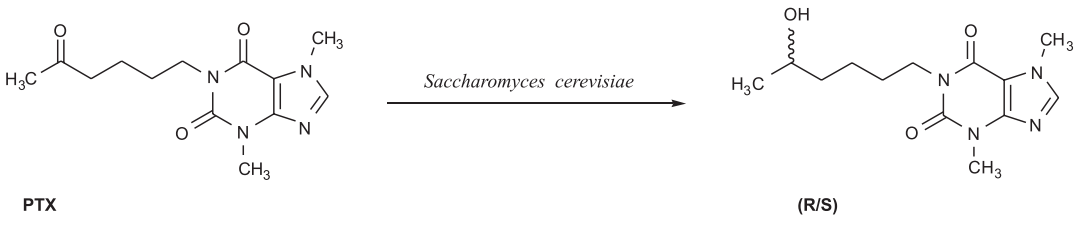

Figure 2. Reduction pathway of pentoxifylline.

Table 1. Bioconversion of **PTX** in water and in organic solvents

Strains	Yields in water		Yields in AcOEt		Yields in hexane		Yields in toluene	
	[%]	ee[%]	[%]	ee[%]	[%]	ee[%]	[%]	ee[%]
KKP13	57	37 (R)	1	60 (S)	nd[b]		1	82 (S)
KKP35	90	85 (R)	——[a]		25	43 (R)	1	98 (S)
KKP82	48	51 (R)	1	58 (S)	——		1	99 (R)
KKP295	65	43 (R)	1	32 (S)	nd		1	100 (R)
KKPU	95	98 (R)	1	68 (S)	——		2	40 (S)
L'hirondelle	45	64 (S)	nd		nd		nd	
Asmussen	68	94 (S)	nd		nd		nd	

[a] ——: biotransformation not observed;
[b] nd: not determined

Table 2. Chromatographic data

Mean values with:	k_1	α	R_S	$-\Delta(\Delta G^O)$ (J · mol^{-1})
SD	6.95 ±0.16	1.171 ± 0.009	2.397 ±0.272	391.4 ±17.7
SEM	6.95 ± 0.06	1.171 ± 0.003	2.397 ± 0.103	391.4 ± 7.5

(k_1) retention factors; (α) separation factors; (R_S) resolution; ($-\Delta(\Delta G^O)$) difference in free energies for enantiomeric separation. The chromatographic conditions: n-hexane/2-propanol 780/220 (v/v); flow rate 1.0 mL/min; column temperature 25°C; UV detection at 275 nm; injection 20 µL.
Mean values for chromatographic data with SD and SEM were determined on the base of results from seven experiments.

$$-\Delta(\Delta G^O) = RT \ln \alpha \quad (4)$$

Polarimetry

The measurements of the optical rotation products of the stereoselective reduction of **PTX** were carried out on a Digital Polarimeter – DIP 2000 (Jasco Inc., Japan). The optical rotation R and S-1-(5-hydroxyhexyl)-3,5-dimethylxanthine were in agreement with the literature values (9,11) {for R enantiomer $[\alpha]_D^{20}$ -5.6° (c=6.7, EtOH); for S enantiomer $[\alpha]_D^{20}$ 5.6° (c=6.7, EtOH)}.

Spectroscopy

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

Chemical synthesis

1-(5-R,S-hydroxyhexyl)-3,5-dimethylxanthine (**HOPTX***)* – racemic standard

HOPTX was synthesized from **PTX** using sodium borohydride reduction. Briefly, 140 mg (0.5 mmol) of **PTX** 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 confirmed by thin layer chromatography (benzene : acetone 1:1, v/v). 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. 109-111°C; 92% yield; HPLC: t_r = 27.50; 32.00 min; IR (KBr) cm $^{-1}$: 3374 (OH), 1698 (CO), 1654 (CO); ^{1}H NMR 300 MHz [$DMSOd_6$]: d [ppm]: 1.00 (d, *J* = 6.0 Hz, 3H, C$\underline{H}_3$-CH), 1.26 (m, 4H, CH_2-CH_2), 1.46 (m, 2H, C$\underline{H}_2$-CHOH), 3.40 (s, 3H, N-CH_3), 3.53 (m, 1H, C$\underline{H}$), 3.80 (t, *J* = 7.4 Hz, 2H, N-CH_2), 3.85 (s, 3H, N-CH_3), 4.30 (s, 1H, OH), 7.97 (s, 1H, Im-H)

Microbiological methods

Biotransformation of **PTX** in an aqueous medium

In a typical experiment 2 g of wine yeast (KKP) or 5 g of baker's yeast was suspended in of 150 mL phosphate buffer. After stirring for 30 min at 30°C, 5 g of glucose and 117 mg (0.5 mmol) of **PTX** were added. After stirring for 5 days at 30°C the yeasts were filtered off. The filtrate was extracted three times with 100 mL methylene chloride and dried over Na_2SO_4. The residues obtained from the evaporation of the solvent were dissolved in 2 mL of 2-propanol for HPLC analysis or purified by silica gel chromatography.

Biotransformation of **PTX** in organic media

1 g of dry baker's or wine yeast was suspended in 25 mL of the organic solvent and 2 mL of tridistilled water was added. After the addition of 58 mg (0.25 mmol) of **PTX** 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.

RESULTS AND DISCUSSION

1-(5-Oxohexyl)-3,5-dimethylxanthine (**PTX**) was reduced with $NaBH_4$ to the racemic standard – R,S-1-(5-hydroxyhexyl)-3,5-dimethylxanthine (**HOPTX**) – which was used as the reference agent in the further analytical research.

The aim of this study was to find a strain of *Saccharomyces cerevisiae* yielding enantiomerically pure (R-1-(5-hydroxyhexyl)-3,5-dimethylxanthine (**LSF**)) from **PTX**. The yeast used for the microbiological reduction was employed under non-fermenting conditions. In Table 1 a summary of the results obtained using aqueous and organic media is given. **PTX** was reduced by baker's yeast (*Saccharomyces cerevisiae*: strains – L'hirondelle and Asmussen) in a water medium according to Prelog's rule (21) to give the (S)-alcohol in good optical purities, ee 94% for Asmussen and 64 % for L'hirondelle. Long-chain ketones such as n-propyl-n-butyl ketone and several bulky phenol ketones are not accepted. Only one long alkyl chain is tolerated, as long as the other substituent is the methyl group (22, 23). However, **LSF** corresponds to the (R)-alcohols and therefore for the following experiments some strains of wine yeast were used as a biocatalyst. Bioreductions were performed in water and organic solvents. Table 1 shows that in numerous cases such reductions can be carried out quite successfully. **PTX** was reduced in the presence of wine yeast in an aqueous medium according to *anti*- Prelog's rule to give a similar (R)-alcohol to that obtained in reduction with LKADH (alcohol dehydrogenase from *Lactobacillus kefiri*) (24, 25). The biotransformation of **PTX** with wine yeasts in water produced LSF with different enantiomeric excesses (ee from 37 % for KKP13 to 98 % for KKPU) and different yields (from 48 % for KKP82 to 95 % for KKPU). The biotransformation process with KKPU was characterised by a high enantioselectivity (ee = 98%) for the R isomer and a high yield (95%). The KKPU strain was proved to have the best **LSF** formation activity. However, in organic media the yields of the reductions of **PTX** into **LSF** were very small (from 1 to 23%). In ethyl acetate all wine strains prefered to form an (S) isomer, whereas in the toluene strains KKP82 and KKP295 (R) isomers were formed. Drastic solvent dependent ee changes were found for the reduction of **PTX** by the KPP35 strain: 85 % ee in favor of the (R) enantiomer in water and 98 % ee favoring (S) enantiomer in toluene. Therefore, the reduction of **PTX** with wine yeast can be performed in organic solvents only in ethyl acetate and toluene, but with low yield. When hexane was used for KKP82 and KKPU the biotransformation of **PTX** was not observed.

In summary, these results demonstrate that the reduction of **PTX** in the water medium with the KKPU strain offers an alternative new and highly selective method of **LSF** formation.

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