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Alcohol Dehydrogenases as Tools for the Preparation of Enantiopure Metabolites of Drugs with Methyl Alkyl Ketone Moiety

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Abstract

Three dehydrogenases – *(R)*-alcohol dehydrogenase from *L. kefir*, *(S)*-aromatic alcohol dehydrogenase from *T.* sp. and *(S)*-alcohol dehydrogenase from *T. brockii* – were tested for the preparation of enantiopure hydroxyl metabolites of pentoxifylline (PTX), propentofylline (PPT) and denbufylline (DBF). These metabolites have an important pharmacological significance. The experimental conditions were optimized for biocatalytic reactions. *LK*ADH produced the chiral secondary alcohols: (*R*)-OHPTX, (*R*)-OHPPT and (*R*)-OHDBF, in an anti-Prelog's rule configuration. In contrast, *TB*ADH and SAADH also generated chiral secondary alcohols, but according to Prelog's rule, giving (*S*)-OHPTX, (*S*)-OHPPT and (*S*)-OHDBF respectively. All the ADHs tested were characterized by a high enantioselectivity (*ee*s of 99–100%), but the yield of bioconversion was only satisfactory for the reactions performed using *LK*ADH, being in the 96–98% range for PPT and PTX respectively.

Keywords

Alcohol dehydrogenases • Stereoselective bioreduction • Pentoxifylline • Propentofylline • Denbufylline

Introduction

Enzymes are being used ever more widely in industrial synthetic chemistry as excellent catalysts. Enzymes allow the omission not only of the tedious blocking and deblocking

steps that are common in enantioselective and regioselective organic synthesis, but also avoid the waste of fossil fuels required for preparing high-temperature and high-pressure consolidations. Thus, from the economic and environmental point of view, enzymes are considered more satisfactory than conventional catalysts.

Chiral alcohols are very useful materials in the specialty chemical and pharmaceutical industries. Enantiomerically pure alcohols are valuable chiral building blocks for industrial fine chemicals. For example, the compounds act as key intermediates in the production of pharmaceuticals, fine chemicals and natural products. Examples of pharmaceuticals with chiral alcohols as intermediates are antihypertensive drugs, calcium and potassium channel blocking drugs, antiarrhythmic agents, β_3-receptor agonists, anticholesterol and antiviral drugs.The interconversion of a ketone to the corresponding chiral alcohol and vice versa represents one of the most common redox-reactions in organic chemistry. Whereas traditional synthetic methods predominantly use toxic metals and expensive complex hydrides, biotransformations offer some significant advantages. Various chiral alcohols can be produced by biocatalysis using two methods: kinetic resolution of the racemic starting material [1, 2], or direct synthesis from a prochiral compounds [3–6]. The asymmetric reduction of prochiral carbonyl substrate is one example of direct synthesis. The vast majority of dehydrogenases and reductases used for ketone reduction and alcohol oxidation require nicotinamide cofactors, such as NADH and NADPH.

For the synthesis of chiral alcohols, commercially available alcohol dehydrogenases (ADHs) isolated from yeast (NADH dependent YADH), horse liver (NADH dependent HLADH) or *Thermoanaerobium brockii* (NADPH-dependent *TB*ADH) can be used for different substrate structures. Horse-liver ADH can be used for the reduction of a broad range of cyclic ketones and 2- or 3-ketoesters [7], while open-chain methyl and ethyl ketones are the preferred substrates for *T. brockii* ADH [8]. An NADPH-dependent ADH from *Rhodococcus erythropolis* (*RE*ADH) was found that reduces a broad variety of ketones with specific activity, giving *(S)*-alcohols [9, 10]. Furthermore, an NADPH-dependent ADH was found in *Lactobacillus* that converted similar ketone structures but formed *(R)*-alcohols [11–13]. *Lactobacillus kefiri* produces an *(R)*-ADH (*LK*ADH) that accepts a broad variety of ketone substrates – including acetophenone and derivatives (ring halogenated), aliphatic, open-chain ketones, 2-, 3-ketoesters, and cyclic ketones – with a high specific activity. In the majority of cases enzymatic and microbial reductions of the alkyl aryl ketones proceed according to Prelog's rule [14, 15] generating alcohols in the *(S)*-configuration.The majority of enzymes, such as HLADH, YADH, *TB*ADH and ADH from *R. erythropolis*, follow this rule, while only a few (e.g. *LK*ADH) have been described as possessing enzymes of the opposite specificity, *i.e*. anti-Prelog's specificity [16].

In this study we present the results of the enantioselective bioreduction of drugs *i.e*. pentoxifylline (**PTX**), propentofylline (**PPT**) and denbufylline (**DBF**) (Fig. 1) using the commercially available dehydrogenases: *(R)*-alcohol dehydrogenase from *L. kefir*, *(S)*-aromatic alcohol dehydrogenase from *T.* sp. and *(S)*-alcohol dehydrogenase from *T. brockii*. The drugs tested possess a methyl ketone moiety in their structures which was reduced biocatalytically by alcohol dehydrogenases. The chiral products obtained are important, pharmacologically significant metabolites [17-23]. For example *(R)*-hydroxy metabolite of **PTX** (*(R)*-**OHPTX**), known as lisofylline, is a drug candidate that has been under investigation for acute respiratory distress syndrome (ADRS), acute lung injury (ALI), septic shock, and mucositis. Moreover, it may prevent neutropenic infections in

cancer patients receiving high dose chemotherapy [24], and it has also been found to be effective in the prevention and treatment of Type 1 diabetes [25]. In contrast, the enantiomeric antipode of lisofylline (*(S)*-**OHPTX**) is considered to be pharmacologically inactive. Studies have shown also, that the racemic mixture and the stereoisomers of **OHPPT** demonstrates 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 transport as effectively as propentofylline. Since inhibition of adenosine transport appears to be important for the neuroprotective effect of **PPT**, the hydroxy metabolites may also provide neuroprotection [26].

Results and Discussion

Configuration

The bioconversion of **PTX**, **PPT** and **DBF** (Fig. 1) was carried out using three commercially-available alcohol dehydrogenases. One of them, *LK*ADH, produced the chiral secondary alcohols, (*R*)-**OHPTX**, (*R*)-**OHPP**T and (*R*)-**OHDBF**, through enantioselective bioreduction, giving an anti-Prelog's rule configuration. The two remaining dehydrogenases, *TB*ADH and SAADH, also generated chiral secondary alcohols, but according to Prelog's rule, giving (*S*)-**OHPTX**, (*S*)-**OHPPT** or (*S*)-**OHDBF**, respectively. The product configuration and the enantioselectivity of the enzymatic reduction were determined using chiral HPLC comparison of the reduction product with the reference agents which we had previously obtained [27–29].

Bioreduction conditions

In all the assays the substrate concentration was kept in the 2.5–4.0 mM range. Bioreductions were performed under different conditions: pH 7.0–7.5, cofactor concentration 1.25–2.0 mM, cofactor regeneration system: 2-propanol 6-8 % v/v. The secondary alcohol, 2-propanol, performs a dual role: firstly, it serves to recycle the cofactor, and secondly it improves the solubility of the substrate in the aqueous medium. **DBF** was poorly soluble in the reaction mixture in comparison to **PTX** or **PPT**. Unfortunately, even the 2-propanol used to recycle the NADP did not improve the solubility of **DBF**. In this case, therefore, it was necessary to use a cosolvent, DMSO (2–2.5%), to ensure an appropriate solubility of **DBF** in the reaction medium. The results are summarized in table 1. All the ADHs tested displayed enantioselective activity (*ee* 99–100%), but only the yield of bioconversion of the reactions performed using *LK*ADH was satisfactory, in the 96–98% range. For *TB*ADH and SAADH the yield was very low and reached a level of only 5–10%.

Unsatisfactory results were observed for bioreductions using SAADH. This enzyme was characterized by a high enantioselectivity for **PTX** and **PPT** but a very low yield of bioconversion, reaching at maximum level of 5%. SAADH was inactive in regard to **DBF**. Not even a trace was obtained of a **DBF** bioconversion product.

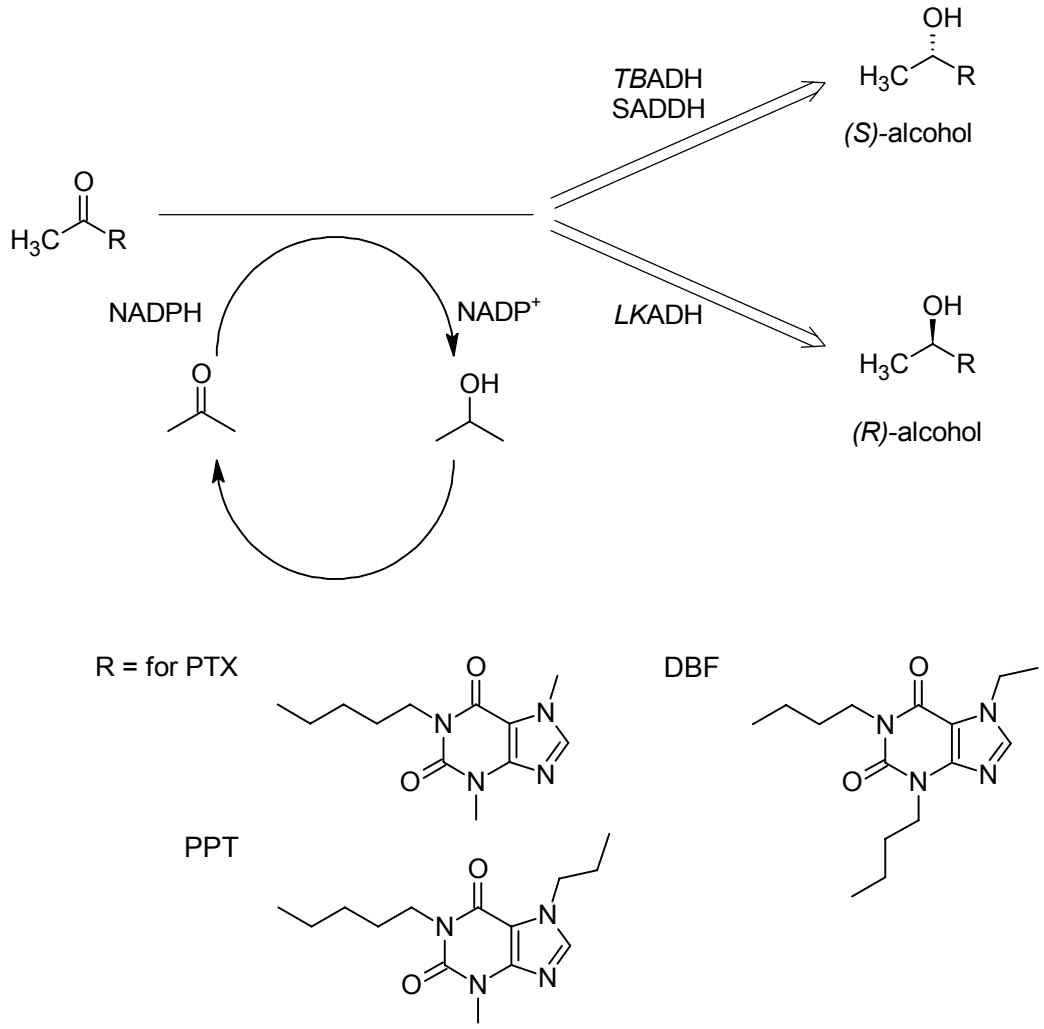

Fig. 1. The bioreduction pathway of xanthine with methyl alkyl ketone moiety.

Tab. 1. Bioreduction of PTX, PPT and DBF with different ADHs.

ADHs	Substrate	Product	HPLC yield (%)	*ee* (%)
*LK*ADH	PTX	*R*-OHPTX	96	>99
	PPT	*R*-OHPPT	98	>99
	DBF	*R*-OHDBF	11	>99.9
*TB*ADH	PTX	*S*-OHPTX	10	100
	PPT	*S*-OHPPT	9	100
	DBF	*S*-OHDBF	8	>99.9
SAADH	PTX	*S*-OHPTX	5	>99.9
	PPT	*S*-OHPPT	5	>99.9
	DBF	—	0	—

The results presented in this paper show that commercially available dehydrogenases can be tools for the preparation of enantiopure hydroxyl metabolites of **PTX**, **PPT** and **DBF**. The highest activity with regard to the drugs tested was shown by *LK*ADH. The tested

agents were converted by this enzyme to appropriate (*R*)-hydroxy metabolites with a high yield. The procedure using *LK*ADH can be an alternative to the whole cell methodology using *Lactobacillus kefiri*, which we previously described (Table 2) [11, 28]. Using whole cells of *L. kefiri* DMS 20587 as a source for (*R*)-specific ADH we obtained (*R*)-**OHPTX**, (*R*)-**OHPPT** and (*R*)-**OHDBF** with a biotransformation yield in the 98-100% range and *ee* values in the 95–98% range. Accordingly, the procedure using *LK*ADH should be employed in cases where the highest enantiopurity is necessary *e.g.* in pharmaco-kinetic/pharmacodynamic studies [23]. The other enzymes studied, *TB*ADH and SAADH, displayed a high enantioselectivity, but unfortunately a low yield of bioconversion (5–10%). DBF was not found in the substrate board of SAADH as no bioreduction process was observed to take place.

Tab. 2. Comparative data for the bioreduction of the drugs tested when using the whole cell methodology.

Drugs	Biocatalyst	yield (%)	ee (%) (configuration)
PTX	*L. kefiri*	100	98 (*R*) [11]
	Yeast	68	94 (*S*) [29]
PPT	*L. kefiri*	98	96 (*R*)[a]
	Yeast	67	78 (*S*) [27]
DBF	*L. kefiri*	98	95 (*R*) [28]
	Yeast	48	72 (*S*)[a]

[a] unpublished data.

From analysis of the data presented for bioreduction using the whole cell methodology with yeast (Table 2) [27, 29] it follows that it would be possible to obtain (*S*)-**OHPTX**, (*S*)-**OHPPT** and (*S*)-**OHDBF** with biotransformation yields in the 48–68% range and with *ee* values in the 72–94% range. In all the cases of bioreduction conducted using commercially-available ADHs it was necessary to make use of a very expensive cofactor: NADPH. This is a very interesting methodology, but from an economic point of view is expensive and less attractive.

Experimental

Chemicals

Some of the drugs used in this research were obtained as gifts from pharmaceutical companies (pentoxifylline from Polpharma, Poland; propentofylline from Intervet, Gemany), while the denbufylline comes from the University of Bonn, Germany. Alcohol dehydrogenase from *L. kefir* (*LK*ADH), *(S)*-aromatic alcohol dehydrogenase from *T.* sp., alcohol dehydrogenase from *T. brockii* (*TB*ADH) and NADPH were purchased from Sigma-Aldrich, USA. The HPLC grade dichloromethane, *n*-hexane, and 2-propanol were from Merck, Germany. All the other chemicals were of analytical reagent grade and were also obtained from Merck and Fluka, Germany.

Enzyme Assays

*LK*ADH assay: 10 mg of NADPH, 660 µL (8 %) of 2-propanol, 0.02 mmol of an appropriate ketone substrate and 20 mg of *LK*ADH (~1 U/mL) were added to a flask containing 8 mL of 50 mM phosphate buffer (pH 7). In the case of **DBF**, which was insoluble in water, 200 µl DMSO (2.5 %) was used as a cosolvent. The mixtures were then incubated for 36 h at 25 °C and 180 rpm. Samples (200 µL) were drawn at regular time intervals. The aqueous layer was extracted using dichloromethane, 3 x 200 µL. The combined and dried (Na_2SO_4) organic layers were evaporated to a residue which was then dissolved in 2-propanol and analyzed using HPLC.

*TB*ADH assay: 10 mg of NADPH, 500 µL of 2-propanol, 0.02 mmol of an appropriate ketone substrate and 20 mg of *TB*ADH (~4.5 U/ mL) were added to a flask containing 5 mL of 100 mM phosphate buffer (pH 7.8). In the case of **DBF**, which was insoluble in water, 100 µL DMSO (2 %) was used as a cosolvent. The mixtures were then incubated for 48 h at 40 °C and 180 rpm. Samples (200 µl) were drawn at regular time intervals. The aqueous layer was extracted using dichloromethane, 3 x 200 µl. The combined and dried (Na_2SO_4) organic layers were evaporated to a residue which was then dissolved in 2-propanol and analyzed using HPLC.

SAADH assay: 10 mg of NADPH, 500 µL of 2-propanol, 0.02 mmol of an appropriate ketone substrate (**PTX** or **PPT**) and ~1 U/ mL of SAADH were added to a flask containing 5 mL of 100 mM phosphate buffer (pH 7.8). The mixtures were then incubated for 48 h at 40 °C and 180 rpm. Samples (200 µl) were drawn at regular time intervals. The aqueous layer was extracted using dichloromethane, 3 x 200 µl. The combined and dried (Na_2SO_4) organic layers were evaporated to a residue which was then dissolved in 2-propanol and analyzed using HPLC.

HPLC analysis

The yields of transformation and enantiomeric excess (ee) were determined using HPLC analysis on a Chiralpak 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 (Dionex UV/VIS detector UVD 170S/340S) set at 275 nm. The analytical chiral column was a 250 mm x 4.6 mm i.d. Daicel Chiralpak AD (Chemical Industries, France), protected with a 20 mm x 4.6 mm LC-Si guard-column (Supelco, Inc., Bellfonte, PA, USA). The temperature was set at 25 ^{o}C. The mobile phase, consisting of n-hexane/ and 2-propanol (78:22 v/v, or 92:8 v/v), was vacuum-degassed before use and pumped at a flow rate of 1 ml min^{-1} for **PTX** and **PPT** and their bioreduction products, and 0.5 ml min^{-1} for **DBF** and its bioreduction product. Under these conditions the approximate retention times were: 7-(2-chloroethyl)-1,3-dimethylxanthine (internal standard): 11.80 min; **PTX**: 22.45 min; (*R*)-**OHPTX**: 25.45 min; (*S*)-**OHPTX**: 28.87 min; **PPT**: 16.12 min; (*R*)-**OHPPT**: 14.25 min; (*S*)-**OHPPT**: 18.20 min; **DBF**: 35.98 min, *(R)*-**OHDBF**: 25.45 min; *(S)*-**OHDBF**: 28.87 min. No interference was observed at the retention times in question.

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Authors' Statement

Competing Interests

The authors declare no conflict of interest.

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